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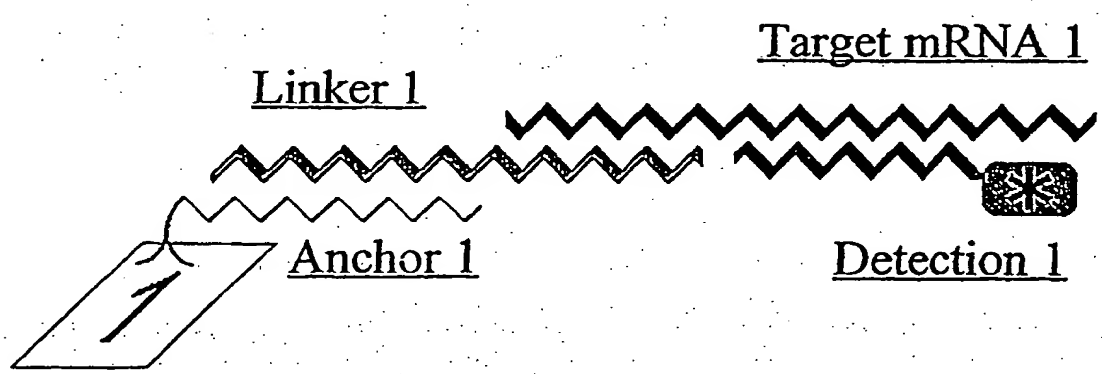

(57) Abstract

The present invention relates to compositions, apparatus and methods useful for concurrently performing multiple, high throughput, biological or chemical assays, using repeated arrays of probes. A combination of the invention comprises a surface, which comprises a plurality of test regions, at least two of which, and in a preferred embodiment, at least twenty of which, are substantially identical, wherein each of the test regions comprises an array of generic anchor molecules. The anchors are associated with bifunctional linker molecules, each containing a portion which is specific for at least one of the anchors and a portion which is a probe specific for a target of interest. The resulting array of probes is used to analyze the presence or test the activity of one or more target molecules which specifically interact with the probes. In one embodiment of the invention, the test regions (which can be wells) are further subdivided into smaller subregions (indentations, or dimples). In one embodiment of the invention, ESTs are mapped. In another embodiment, the presence of a target nucleic acid is detected by protecting the target against nuclease digestion with a polynucleotide fragment, and analyzing the protected polynucleotide by mass spectrometry.

HIGH THROUGHPUT ASSAY SYSTEM FOR MONITORING ESTS

This application claims priority of provisional application 60/068,291, filed December 19, 1997, and of U.S. application of serial number 09/109,076, filed on July 2, 1998, each of which disclosure is incorporated by reference herein.

Background Of The Invention

This invention relates, *e.g.*, to compositions, apparatus and methods useful for concurrently performing multiple biological or chemical assays, using repeated arrays of probes. A plurality of regions each contains an array of generic anchor molecules. The anchors are associated with bifunctional linker molecules, each containing a portion which is specific for at least one of the anchors and a portion which is a probe specific for a target of interest. The resulting array of probes is used to analyze the presence of one or more target molecules which interact specifically with the probes. The invention relates to diverse fields distinguished by the nature of the molecular interaction, including but not limited to pharmaceutical drug discovery, molecular biology, biochemistry, pharmacology and medical diagnostic technology.

Pluralities of molecular probes arranged on surfaces or "chips" have been used in a variety of biological and chemical assays. Assays are performed to determine if target molecules of interest interact with any of the probes. After exposing the probes to target molecules under selected test conditions, detection devices determine whether a target molecule has interacted with a given probe.

These systems are useful in a variety of screening procedures for obtaining information about either the probes or the target molecules. For example, they have been used to screen for peptides or potential drugs which bind to a receptor of interest, among others; to screen samples for the presence of, for example, genetic mutations, allelic

assay can be run in many (100 for example) 96-well microplates at one time. Each well of a plate can have, *e.g.*, 36 different tests performed in it by using an array of about 36 anchor and linker pairs. That is, 100 plates, with 96 wells per plate, and each with 36 tests per well, can allow for a total of 345,000 tests; for example, each of 9,600 different drug candidates can be tested simultaneously for 36 different parameters or assays. High throughput assays provide much more information for each drug candidate than do assays which test only one parameter at a time. For example, it is possible in a single initial high throughput screening assay to determine whether a drug candidate is selective, specific and/or nontoxic. Non-high throughput methods necessitate extensive follow-up assays to test such parameters for each drug candidate of interest. Several types of high throughput screening assays are described, *e.g.*, in Examples 15-17. The ability to perform simultaneously a wide variety of biological assays and to do very many assays at once (*i.e.*, in very high throughput) are two important advantages of the invention.

In one embodiment, for example, using 96-well DNA Bind plates (Corning Costar) made of polystyrene with a derivatized surface for the attachment of primary amines, such as amino acids or modified oligonucleotides, a collection of 36 different oligonucleotides can be spotted onto the surface of every well of every plate to serve as anchors. The anchors can be covalently attached to the derivatized polystyrene, and the same 36 anchors can be used for all screening assays. For any particular assay, a given set of linkers can be used to program the surface of each well to be specific for as many as 36 different targets or assay types of interest, and different test samples can be applied to each of the 96 wells in each plate. The same set of anchors can be used multiple times to re-program the surface of the wells for other targets and assays of interest, or it can be re-used multiple times with the same set of linkers. This flexibility and reusability represent further advantages of the invention.

One embodiment of the invention is a combination useful for the detection of one or more target(s) in a sample, which comprises, before the addition of said sample,

a) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising

b) at least eight different oligonucleotide anchors, each in association with

c) a bifunctional linker which has a first portion that is specific for the

to the anchor. The second portion, which is a probe specific for the target of interest - here, target mRNA 1 - is in this illustration an oligonucleotide which can hybridize to that target. Although not illustrated in this figure, each of the remaining five anchors can hybridize to its own linker via the anchor-specific portion; each linker can contain a probe portion specific for, *e.g.*, an mRNA different from (or the same as) mRNA 1. This illustrated combination can be used to assay as many as 15 different samples at the same time for the presence of mRNA 1 (or, simultaneously, for mRNA targets which are specified (programmed) by the other five probes in the array). To perform the assay, each sample, which in this example can be an RNA extract from, say, one of 15 independent cell lines, is added in a small volume to one of the regions, or wells, and incubated under conditions effective for hybridization of the probe and the target. In order to determine if mRNA 1 is present in a sample, a detection device which can recognize patterns, and/or can interrogate specific locations within each region for the presence of a signal, is employed. If the cell lines are incubated under conditions in which their mRNAs are labeled *in vivo* with a tag, and if mRNA 1 is present in a sample, the detector will detect a signal emanating from the tagged mRNA at the location defined by anchor/probe complex 1. Alternatively, the mRNA can be directly labeled *in vitro*, before or after being added to the regions (wells). Alternatively, as is illustrated in Fig. 1, mRNA can be tagged indirectly, before or after it has hybridized to the probe, *e.g.*, by incubating the RNA with a tagged "detector" oligonucleotide (target-specific reporter oligonucleotide) which is complementary to a sequence other than that recognized by the probe. In the illustrated example, 15 samples can be analyzed simultaneously. Because at least 20 or more, *e.g.*, as many as 1536 or more, samples can be analyzed simultaneously with this invention, it is a very high throughput assay system.

As used herein, "target" refers to a substance whose presence, activity and/or amount is desired to be determined and which has an affinity for a given probe. Targets can be man-made or naturally-occurring substances. Also, they can be employed in their unaltered state or as aggregates with other species. Targets can be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. Examples of targets which can be employed in this invention include, but are not limited to, receptors (on vesicles, lipids, cell membranes or a variety of other receptors); ligands, agonists or antagonists which bind to specific receptors; polyclonal

inorganic materials or combinations thereof, including, merely by way of example, plastics such as polypropylene or polystyrene; ceramic; silicon; (fused) silica, quartz or glass, which can have the thickness of, for example, a glass microscope slide or a glass cover slip; paper, such as filter paper; diazotized cellulose; nitrocellulose filters; nylon membrane; or polyacrylamide gel pad. Substrates that are transparent to light are useful when the method of performing an assay involves optical detection. In a preferred embodiment, the surface is the plastic surface of a multiwell, *e.g.*, tissue culture dish, for example a 24-, 96-, 256-, 384-, 864- or 1536-well plate (*e.g.*, a modified plate such as a Corning Costar DNA Bind plate). Anchors can be associated, *e.g.*, bound, directly with a surface, or can be associated with one type of surface, *e.g.*, glass, which in turn is placed in contact with a second surface, *e.g.*, within a plastic "well" in a microtiter dish. The shape of the surface is not critical. It can, for example, be a flat surface such as a square, rectangle, or circle; a curved surface; or a three dimensional surface such as a bead, particle, strand, precipitate, tube, sphere; etc.

The surface comprises regions which are spatially discrete and addressable or identifiable. Each region comprises a set of anchors. How the regions are separated, their physical characteristics, and their relative orientation to one another are not critical. In one embodiment, the regions can be separated from one another by any physical barrier which is resistant to the passage of liquids. For example, in a preferred embodiment, the regions can be wells of a multiwell (*e.g.*, tissue culture) dish, for example a 24-, 96-, 256-, 384-, 864- or 1536-well plate. Alternatively, a surface such as a glass surface can be etched out to have, for example, 864 or 1536 discrete, shallow wells. Alternatively, a surface can comprise regions with no separations or wells, for example a flat surface, *e.g.*, piece of plastic, glass or paper, and individual regions can further be defined by overlaying a structure (*e.g*,. a piece of plastic or glass) which delineates the separate regions. Optionally, a surface can already comprise one or more arrays of anchors, or anchors associated with linkers, before the individual regions are delineated. In another embodiment, arrays of anchors within each region can be separated from one another by blank spaces on the surface in which there are no anchors, or by chemical boundaries, such as wax or silicones, to prevent spreading of droplets. In yet another embodiment, the regions can be defined as tubes or fluid control channels, *e.g.*, designed for flow-through assays, as disclosed, for example, in Beattie *et al* (1995). *Clin.*

molecules that can be recognized by a scanning detection device as a "starting point" for aligning the locations of the regions on a surface.

The size and physical spacing of the test regions are not limiting. Typical regions are of an area of about 1 to about 700 mm^2, preferably 1 to about 40 mm^2, and are spaced about 0.5 to about 5 mm apart, and are routinely selected depending on the areas involved. In a preferred embodiment, the regions are spaced approximately 5 mm apart. For example, each region could comprise a rectangular grid, with, for example, 8 rows and 6 columns, of roughly circular spots of anchors which are about 100 micrometers in diameter and 500 micrometers apart; such a region would cover about a 20 millimeter square area. Larger and smaller region areas and spacings are included.

The regions can also be further subdivided such that some or all anchors within a region are physically separated from neighboring anchors by means, *e.g.*, of an indentation or dimple. For example, the number of subdivisions (subregions) in a region can range from about 10 to about 100 or more or less. In one embodiment, a region which is a well of a 1536-well dish can be further subdivided into smaller wells, e.g., about 4 to about 900, preferably about 16 to about 36 wells, thereby forming an array of wells-within-wells. See Fig. 4. Such a dimpled surface reduces the tolerance required for physically placing a single anchor (or group of anchors) into each designated space (locus), and the size of the areas containing anchors is more uniform, thereby facilitating the detection of targets which bind to the probe.

The term "anchor" as used herein refers to any entity or substance, *e.g.*, molecule (or "group" of substantially identical such substances (see, *e.g.*, Fig. 7)) which is associated with (*e.g.*, immobilized on, or attached either covalently or non-covalently to) the surface, or which is a portion of such surface (*e.g.*, derivatized portion of a plastic surface), and which can undergo specific interaction or association with a linker or other substance as described herein. As used herein, an "anchor/linker complex" exists when an anchor and a linker have combined through molecular association in a specific manner. The interaction with the linker can be either irreversible, such as via certain covalent bonds, or reversible, such as via nucleic acid hybridization. In a preferred embodiment, the anchor is a nucleic acid, which can be of any length (*e.g.*, an oligonucleotide) or type (*e.g.*, DNA, RNA, PNA, or a PCR product of an RNA or DNA molecule). The nucleic acid can be modified or substituted (*e.g.*, comprising non

a surface with 384 test regions (*e.g.*, wells). In a most preferred embodiment, each anchor in a test region has a different specificity from every other anchor in the array. However, two or more of the anchors can share the same specificity and all of the anchors can be identical. In one embodiment, in which a combination of the invention comprises a very large number of test regions (*e.g.*, about 864, 1536, or more), so that a large number of test samples can be processed at one time, it might of interest to test those samples for only a limited number (*e.g.*, about 2, 4, 6 or 9) of parameters. In other words, for combinations comprising a very large number of regions, it might be advantageous to have only about 2 to 9 anchors per region.

The physical spacing and relative orientation of the anchors in or on a test region are not limiting. Typically, the distance between the anchors is about 0.003 to about 5 mm or less, preferably between about 0.03 and about 1. Larger and smaller anchor spacings (and areas) are included. The anchors can be arranged in any orientation relative to one another and to the boundaries of the region. For example, they can be arranged in a two-dimensional orientation, such as a square, rectangular, hexagonal or other array, or a circular array with anchors emanating from the center in radial lines or concentric rings. The anchors can also be arranged in a one-dimensional, linear array. For example, oligonucleotides can be hybridized to specific positions along a DNA or RNA sequence to form a supramolecular array. Alternatively, the anchors can be laid down in a "bar-code"-like formation. (See Fig. 6). For example, anchors can be laid down as long lines parallel to one another. The spacing between or the width of each long line can be varied in a regular way to yield a simple, recognizable pattern much like a bar-code, *e.g.*, the first and third lines can be twice as large as the rest, lines can be omitted, etc. An extra empty line can be placed after the last line to demarcate one test region, and the bar code pattern can be repeated in succeeding test regions.

The pattern of anchors does not need to be in strict registry with the positions of the separated assay wells (test regions) or separate assay droplets. The term "assay positions" will be used to refer to the positions of the assay surface where assay samples are applied. (These can be defined by the position of separate droplets of assay sample or by the position of walls or separators defining individual assay wells on a multi-well plate for example.) The anchor pattern itself (*e.g.*, a "bar code"-like pattern of oligonucleotide anchors) is used to define where exactly each separate anchor is

components (*e.g.*, anchor and anchor-specific region of a linker, a probe and its target, or a target and a target-specific reporter) bind selectively to each other and, in the absence of any protection technique, not generally to other components unintended for binding to the subject components. The parameters required to achieve specific interactions can be determined routinely, *e.g.*, using conventional methods in the art.

For nucleic acids, for example, one of skill in the art can determine experimentally the features (such as length, base composition, and degree of complementarity) that will enable a nucleic acid (*e.g.*, an oligonucleotide anchor) to hybridize to another nucleic acid (*e.g.*, the anchor-specific portion of a linker) under conditions of selected stringency, while minimizing non-specific hybridization to other substances or molecules (*e.g.*, other oligonucleotide linkers). Typically, the DNA or other nucleic acid sequence of an anchor, a portion of a linker, or a detector oligonucleotide will have sufficient complementarity to its binding partner to enable it to hybridize under selected stringent hybridization conditions, and the T_m will be about 10° to 20° C. above room temperature (*e.g.*, about 37° C). In general, an oligonucleotide anchor can range from about 8 to about 50 nucleotides in length, preferably about 15, 20, 25 or 30 nucleotides. As used herein, "high stringent hybridization conditions" means any conditions in which hybridization will occur when there is at least 95%, preferably about 97 to 100%, nucleotide complementarity (identity) between the nucleic acids. However, depending on the desired purpose, hybridization conditions can be selected which require less complementarity, *e.g.*, about 90%, 85%, 75%, 50%, etc. Among the hybridization reaction parameters which can be varied are salt concentration, buffer, pH, temperature, time of incubation, amount and type of denaturant such as formamide, etc. (see, *e.g.*, Sambrook *et al.* (1989). *Molecular Cloning: A Laboratory Manual* (2d ed.) Vols. 1-3, Cold Spring Harbor Press, New York; Hames *et al.* (1985). *Nucleic Acid Hybridization*, IL Press; Davis *et al.* (1986), *Basic Methods in Molecular Biology*, Elsevir Sciences Publishing, Inc., New York). For example, nucleic acid (*e.g.*, linker oligonucleotides) can be added to a test region (*e.g.*, a well of a multiwell plate - in a preferred embodiment, a 96 or 384 or greater well plate), in a volume ranging from about 0.1 to about 100 or more µl (in a preferred embodiment, about 1 to about 50 µl, most preferably about 40 µl), at a concentration ranging from about 0.01 to about 5 µM (in a preferred embodiment, about 0.1 µM), in a buffer such as, for example, 6X SSPE-T (0.9

to such anchor but with differing "probe" portions; thus, a single array of generic anchors can be used to program or define a varied set of probes. The flexible nature of such a generic assay of anchors can be illustrated with reference to Figures 1 and 2. Fig. 2 illustrates a surface which comprises 15 test regions, each of which contains an array of 6 different anchors, which in this example can be oligonucleotides. Fig. 1 schematically illustrates one of these (oligonucleotide) anchors, anchor 1, which is in contact with linker 1, which comprises one portion that is specific for anchor 1 and a second portion that is specific for target mRNA 1. Alternatively, one could substitute, *e.g.*, a linker 2, which, like linker 1, comprises a portion that is specific for anchor 1, but which comprises a second portion that is specific for target mRNA 2 instead of target mRNA 1. Thus, anchor 1 can be used to specify (or program, or define, or determine) probes for either of two or more different target mRNAs. The process of generating and attaching a high resolution pattern (array) of oligonucleotides or peptides can be expensive, time-consuming and/or physically difficult. The ability to use a pre-formed array of anchors to program a wide variety of probe arrays is one advantage of this invention.

Although the generic anchors illustrated in Fig. 2 define a pattern of oligonucleotide probes, the identical anchor array could also be used to program an array of other probes, for example receptor proteins (see, *e.g.*, Fig. 3). Clearly, many permutations are possible, given the range of types of anchor/linker interactions, *e.g.*, even more complex layers of "sandwiched" or "piggybacked" probes such as protein/antibody combinations. Thus, the surface of anchors per this invention, itself, offers novel advantages.

In one embodiment of the invention, anchors can interact reversibly with linkers; thus, a generic set of anchors can be re-used to program a varied set of probes. For example, an oligonucleotide anchor can be separated from the oligonucleotide portion of a linker by, for example, a heating step that causes the two oligonucleotides to dissociate, and can then be rebound to a second linker. The ability to re-use anchor arrays, which can be expensive, time-consuming and/or physically difficult to make, is another advantage of the invention.

An anchor does not necessarily have to interact with a linker. For example, an anchor can be coupleu (..rectly or indirectly) to a detectable molecule, such as a fluorochrome, and can thereby serve to localize a spot within a grid, *e.g.*, for purpose of

recognized methods, determine experimentally the features of an oligonucleotide that will hybridize optimally to the target, with minimal hybridization to non-specific, interfering DNA or RNA (*e.g.*, see above). In general, the length of an oligonucleotide probe used to distinguish a target mRNA present in a background of a large excess of untargeted RNAs can range from about 8 to about 50 nucleotides in length, preferably about 18, 20, 22 or 25 nucleotides. An oligonucleotide probe for use in a biochemical assay in which there is not a large background of competing targets can be shorter. Using art-recognized procedures (*e.g.*, the computer program BLAST), the sequences of oligonucleotide probes can be selected such that they are mutually unrelated and are dissimilar from potentially interfering sequences in known genetics databases. The selection of hybridization conditions that will allow specific hybridization of an oligonucleotide probe to an RNA can be determined routinely, using art-recognized procedures (*e.g.*, see above). For example, target RNA [*e.g.*, total RNA or mRNA extracted from tissues or cells grown (and optionally treated with an agent of interest) in any vessel, such as the well of a multiwell microtiter plate (*e.g.*, 96 or 384 or more wells)] can be added to a test region containing a oligonucleotide probe array (see above) in a buffer such as 6X SSPE-T or others, optionally containing an agent to reduce non-specific binding (*e.g.*, about 0.5 mg/ml degraded herring or salmon sperm DNA, or yeast RNA), and incubated at an empirically determined temperature for a period ranging from between about 10 minutes and at least 18 hours (in a preferred embodiment, about 3 hours). The stringency of the hybridization can be the same as, or less than, the stringency employed to associate the anchors with the anchor-specific portion of the linkers. The design and use of other types of probes are also routine in the art, *e.g.*, as discussed above.

The anchor-specific and the target-specific portions of a linker can be joined (attached, linked) by any of a variety of covalent or non-covalent linkages, the nature of which is not essential to the invention. The two portions can be joined directly or through an intermediate molecule. In one embodiment, in which both portions of the linker are oligonucleotides, they can be joined by covalent linkages such as phosphodiester bonds to form a single, colinear nucleic acid. In another embodiment, in which the anchor-specific portion is an oligonucleotide and the target-specific portion is a receptor, for example a receptor protein, the two portions can be joined via the

a thin piece of material, *e.g.*, silicone, shaped in the form of a screen or fine meshwork. The base can be a flat piece of material, *e.g.*, glass, in, for example, the shape of the lower portion of a typical microplate used for a biochemical assay. The top surface of the base can be flat, as illustrated in Fig. 5c, or can be formed with indentations that will align with the subdivider shape to provide full subdivisions, or wells, within each sample well. The three pieces can be joined by standard procedures, for example the procedures used in the assembly of silicon wafers.

Oligonucleotide anchors, linker moieties, or detectors can be synthesized by conventional technology, *e.g.*, with a commercial oligonucleotide synthesizer and/or by ligating together subfragments that have been so synthesized. In one embodiment of the invention, preformed nucleic acid anchors, such as oligonucleotide anchors, can be situated on or within the surface of a test region by any of a variety of conventional techniques, including photolithographic or silkscreen chemical attachment, disposition by ink jet technology, capillary, screen or fluid channel chip, electrochemical patterning using electrode arrays, contacting with a pin or quill, or denaturation followed by baking or UV-irradiating onto filters (see, *e.g.*, Rava *et al* (1996). U.S. Patent No. 5,545,531; Fodor *et al* (1996). U.S. Patent No. 5,510,270; Zanzucchi *et al* (1997). U.S. Patent No. 5,643,738; Brennan (1995). U.S. Patent No. 5,474,796; PCT WO 92/10092; PCT WO 90/15070). Anchors can be placed on top of the surface of a test region or can be, for example in the case of a polyacrylamide gel pad, imbedded within the surface in such a manner that some of the anchor protrudes from the surface and is available for interactions with the linker. In a preferred embodiment, preformed oligonucleotide anchors are derivatized at the 5' end with a free amino group; dissolved at a concentration routinely determined empirically (e.g., about 1 μM) in a buffer such as 50 mM phosphate buffer, pH 8.5 and 1 mM EDTA; and distributed with a Pixus nanojet dispenser (Cartesian Technologies) in droplets of about 10.4 nanoliters onto specific locations within a test well whose upper surface is that of a fresh, dry DNA Bind plate (Corning Costar). Depending on the relative rate of oligonucleotide attachment and evaporation, it may be required to control the humidity in the wells during preparation. In another embodiment, oligonucleotide anchors can be synthesized directly on the surface of a test region, using conventional methods such as, *e.g.*, light-activated deprotection of growing oligonucleotide chains (*e.g.*, in conjunction with the use of a site

2) at least 8 different oligonucleotide anchors,

c) contacting said first hybridization product or said second hybridization product with a labeled detector probe, and

d) detecting said detection probe.

Each of the assays or procedures described below can be performed in a high throughput manner, in which a large number of samples (*e.g.*, as many as about 864, 1036, 1536, 2025 or more, depending on the number of regions in the combination) are assayed on each plate or surface rapidly and concurrently. Further, many plates or surfaces can be processed at one time. For example, in methods of drug discovery, a large number of samples, each comprising a drug candidate (*e.g.*, a member of a combinatorial chemistry library, such as variants of small molecules, peptides, oligonucleotides, or other substances), can be added to separate regions of a combination as described or can be added to biological or biochemical samples that are then added to separate regions of a combination, and incubated with probe arrays located in the regions; and assays can be performed on each of the samples. With the recent advent and continuing development of high-density microplates, DNA spotting tools and of methods such as laser technology to generate and collect data from even denser microplates, robotics, improved dispensers, sophisticated detection systems and data-management software, the methods of this invention can be used to screen or analyze thousands or tens of thousands or more of compounds per day.

For example, in embodiments in which the probes are oligonucleotides, the assay can be a diagnostic nucleic acid or polynucleotide screen (*e.g.*, a binding or other assay) of a large number of samples for the presence of genetic variations or defects (*e.g.*, polymorphisms or specific mutations associated with diseases such as cystic fibrosis. See, *e.g.*, Iitia *et al* (1992). *Molecular and Cellular Probes* 6, 505-512)); pathogenic organisms (such as bacteria, viruses, and protozoa, whose hosts are animals, including humans, or plants), or mRNA transcription patterns which are diagnostic of particular physiological states or diseases. Nucleic acid probe arrays comprising portions of ESTs (including full-length copies) can be used to evaluate transcription patterns produced by cells from which the ESTs were derived (or others). Nucleic acid probes can also detect peptides, proteins, or protein domains which bind specifically to particular nucleic acid

linked assays can be performed in such an array format. Combinations of the invention can also be used to detect mutant enzymes, which are either more or less active than their wild type counterparts, or to screen for a variety of agents including herbicides or pesticides.

Of course, MAPS assays can be used to quantitate (measure, quantify) the amount of active target in a sample, provided that probe is not fully occupied, that is, not more than about 90% of available probe sites are bound (or reacted or hybridized) with target. Under these conditions, target can be quantitated because having more target will result in having more probe bound. On the other hand, under conditions where more than about 90% of available probe sites are bound, having more target present would not substantially increase the amount of target bound to probe. Any of the heretofore-mentioned types of targets can be quantitated in this manner. For example, Example 6 describes the quantitation of oligonucleotide targets. Furthermore, it demonstrates that even if a target is present in large excess (*e.g.*, if it is present in such large amounts that it saturates the amount of available probe in a MAPS probe array), by adding known amounts of unlabeled target to the binding mixture, one can "shift the sensitivity" of the reaction in order to allow even such large amounts of target to be quantitated.

In another embodiment, combinations of the invention can be used to screen for agents which modulate the interaction of a target and a given probe. An agent can modulate the target/probe interaction by interacting directly or indirectly with either the probe, the target, or a complex formed by the target plus the probe. The modulation can take a variety of forms, including, but not limited to, an increase or decrease in the binding affinity of the target for the probe, an increase or decrease in the rate at which the target and the probe bind, a competitive or non-competitive inhibition of the binding of the probe to the target, or an increase or decrease in the activity of the probe or the target which can, in some cases, lead to an increase or decrease in the probe/target interaction. Such agents can be man-made or naturally-occurring substances. Also, such agents can be employed in their unaltered state or as aggregates with other species; and they can be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance. For example, to identify potential "blood thinners," or agents which interact .. one of the cascade of proteases which cause blood clotting, cocktails of the proteases of interest can be tested with a plurality of candidate agents and

example, a series of cells (*e.g.*, from a disease model) can be contacted with a series of agents (*e.g.*, for a period of time ranging from about 10 minutes to about 48 hours or more) and, using routine, art-recognized methods (*e.g.*, commercially available kits), total RNA or mRNA extracts can be made. If it is desired to amplify the amount of RNA, standard procedures such as RT-PCR amplification can be used (see, *e.g.*, Innis *et al* eds., (1996) *PCR Protocols: A Guide to Methods in Amplification*, Academic Press, New York). The extracts (or amplified products from them) can be allowed to contact (*e.g.*, incubate with) a plurality of substantially identical arrays which comprise probes for appropriate indicator RNAs, and those agents which are associated with a change in the indicator expression pattern can be identified. Example 15 describes a high throughput assay to screen for compounds which may alter the expression of genes that are correlative with a disease state.

Similarly, agents can be identified which modulate expression patterns associated with particular physiological states or developmental stages. Such agents can be man-made or naturally-occurring substances, including environmental factors such as substances involved in embryonic development or in regulating physiological reactions, or substances important in agribusiness such as pesticides or herbicides. Also, such agents can be employed in their unaltered state or as aggregates with other species; and they can be attached, covalently or noncovalently, to a binding member, either directly or via a specific binding substance.

Another embodiment of the invention is a kit useful for the detection of at least one target in a sample, which comprises:

a) a surface, comprising multiple spatially discrete regions, at least two of which are substantially identical, each region comprising at least eight different anchors (oligonucleotide, or one of the other types described herein), and

b) a container comprising at least one bifunctional linker molecule, which has a first portion specific for at least one of said anchor(s) and a second portion that comprises a probe which is specific for at least one of said target(s).

In one embodiment, there is provided a surface as in a)above and a set of instructions for attaching to at least one of said anchors a bifunctional linker molecule, which has a first portion specific for at least one of said anchor(s) and a second portion that comprises a probe which is specific for at least one target. The instructions can

than) the number of ESTs to be mapped are distributed in separate regions (*e.g.*, wells) of a surface; in the illustrated example, the surface of the combination comprises 16 wells, each of which contains an array of 16 different EST-specific oligonucleotides, numbered 1-16. An oligonucleotide which "corresponds to" an EST (is "EST-specific") is one that is sufficiently complementary to an EST such that, under selected stringent hybridization conditions, the oligonucleotide will hybridize specifically to that EST, but not to other, unrelated ESTs. An EST-corresponding oligonucleotide of this type can bind specifically (under optimal conditions) to the coding or non-coding strand of a cDNA synthesized from the gene from which the EST was originally generated or to an mRNA synthesized from the gene from which the EST was originally generated. Factors to be considered in designing oligonucleotides, and hybridization parameters to be optimized in order to achieve specific hybridization, are discussed elsewhere in this application. In order to assemble the arrays, linker molecules are prepared, each of which comprises a moiety specific for one of the anchors of a generic array plus a moiety comprising an oligonucleotide probe that corresponds to one of the ESTs to be mapped; and the linkers are attached to anchors as described elsewhere in this application. In a subsequent step, an aliquot of a sample comprising a mixture of nucleic acids (*e.g.*, mRNA or single stranded or denatured cDNA), which may contain sequences that are complementary to one or more of the oligonucleotide probes, is added to each of the regions (wells) which comprises a probe array; the mixture is then incubated under routinely determined optimal conditions, thereby permitting nucleic acid to bind to complementary probes. If several of the EST-specific probes are complementary to different portions of a single nucleic acid, that nucleic acid will bind to each of the loci in the array at which one of those probes is located.

In a subsequent step, a different detector oligonucleotide (in the illustrated example, detectors #1 to 16) is added to each region (well) (see Fig. 19). A detector oligonucleotide is designed for each of the ESTs to be mapped. Each EST-specific detector corresponds to a different (at least partially non-overlapping) portion of the EST than does the probe oligonucleotide, so that the probe and the detector oligonucleotides do not interfere with one another. Consider, for example, the ESTs depicted in Figure 21, which correspond to ESTs 1, 2 and 6 of Figures 18-20. Figure 21 indicates that ESTs #1 and #2 were both obtained from gene X (they are "linked"), whereas EST #6 was

c) detecting which oligonucleotide probes of said array are labeled by said detector oligonucleotide,

wherein said array of oligonucleotide probes is immobilized on a region of a combination, wherein said combination comprises

1) a surface comprising a number of spatially discrete, substantially identical, regions equal to the number of ESTs to be studied, each region comprising

2) a number of different anchors equal to the number of ESTs to be studied, each anchor in association with

3) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises an oligonucleotide probe which corresponds to at least one of said ESTs.

In another aspect, the invention relates to a method as above, wherein one or more of said ESTs may be complementary to said nucleic acid, and wherein each of said ESTs comprises two different oligonucleotide sequences, the first of which defines an oligonucleotide probe corresponding to said EST, and the second of which defines a detector oligonucleotide corresponding to said EST, comprising,

a) contacting a sample which comprises molecules of said nucleic acid with at least one region of a combination, wherein said region comprises an array of oligonucleotide probes, at least one of which corresponds to each of said ESTs,

b) incubating said sample with said region, thereby permitting molecules of said nucleic acid to bind to said EST-corresponding oligonucleotide probes which are complementary to portions of said nucleic acid,

c) incubating said region comprising molecules of said nucleic acid bound to one or more of said EST-corresponding oligonucleotide probes with a detector oligonucleotide which corresponds to an EST to which a given one of the oligonucleotide probes of said array corresponds, thereby binding detector oligonucleotides to nucleic acid molecules which have bound to said given oligonucleotide probe or to other oligonucleotide probes which are complementary to said nucleic acid,

d) detecting the presence of said detector oligonucleotides, thereby identifying which EST-corresponding oligonucleotide probes of said array are complementary to portions of a nucleic acid which binds to said given oligonucleotide EST-corresponding probe, thereby identifying which ESTs are complementary to said

combination, wherein said combination comprises

1) a surface comprising a number of spatially discrete, substantially identical, regions equal to the number of ESTs to be studied, each region comprising

2) a number of different anchors equal to the number of ESTs to be studied.

Of course, the above methods for mapping ESTs can be used to map test sequences (*e.g.*, polynucleotides) onto any nucleic acid of interest. For example, one can determine if two or more cloned DNA fragments or cDNAs map to the same genomic DNA. Such a procedure could aid, for example, in the structural elucidation of long, complex genes. In a similar manner, one can determine if one or more spliced out sequences or coding sequences map to the same genomic DNA. Such a determination could be used, for example, in a diagnostic test to distinguish between a normal and a disease condition which are characterized by differential splicing patterns. Many other applications of the mapping method will be evident to one of skill in the art.

In another aspect, the invention relates to a method of determining which of a plurality of polynucleotides are complementary to a given nucleic acid,

wherein one or more of said polynucleotides may be complementary to said nucleic acid, and wherein each of said polynucleotides comprises two different oligonucleotide sequences, the first of which defines an oligonucleotide probe corresponding to said polynucleotide, and the second of which defines a detector oligonucleotide corresponding to said polynucleotide, comprising,

a) contacting a sample which comprises molecules of said nucleic acid with at least one region of a combination, wherein said region comprises an array of oligonucleotide probes, at least one of which corresponds to each of said polynucleotides,

b) incubating said sample with said region, thereby permitting molecules of said nucleic acid to bind to said polynucleotide-corresponding oligonucleotide probes which are complementary to portions of said nucleic acid,

c) incubating said region comprising molecules of said nucleic acid bound to one or more of said polynucleotide-corresponding oligonucleotide probes with a detector oligonucleotide which corresponds to a polynucleotide to which a given one of the oligonucleotide probes of said array corresponds, thereby binding detector oligonucleotides to nucleic acid molecules which have bound to said given oligonucleotide probe or to other oligonucleotide probes which are complementary to

ribonucleases or by chemical procedures, such as treatment with alkali, leaving behind the single strand cDNA, which is subsequently placed in contact with a MAPS probe array. The use of reverse transcriptase in this method minimizes the need for extensive handling of RNA, which can be sensitive to degradation by nucleases and thus difficult to work with. Furthermore, the additional specificity engendered by the specific reverse transcriptase primers imparts an added layer of specificity to the assay.

Optionally, the cDNAs described above can be amplified before hybridization to the probe array to increase the signal strength. The oligonucleotide reverse transcriptase primers described above can comprise, at their 5' ends, sequences (which can be about 22-27 nucleotides long) that specify initiation sites for an RNA polymerase (*e.g.*, T7, T3 or SP2 polymerase, or the like). In the example shown in Figure 8, a T7 promoter sequence has been added to the reverse transcriptase primer. The polymerase recognition site becomes incorporated into the cDNA and can then serve as a recognition site for multiple rounds of transcription by the appropriate RNA polymerase (*in vitro* transcription, or IVT). Optionally, the mRNAs so generated can be amplified further, using PCR and appropriate primers, or the cDNA, itself, can be so amplified. Procedures for transcription and PCR are routine and well-known in the art.

The above-described method, in which mRNA targets are converted to cDNA with reverse transcriptase before assaying on MAPS plates, can be used instead of the standard MAPS assay procedure for any of the RNA-based assays described above.

In another embodiment of the invention, one or more nucleic acid targets of interest are hybridized to specific polynucleotide protection fragments and subjected to a nuclease protection procedure, and those protection fragments which have hybridized to the target(s) of interest are assayed on MAPS plates. If the target of interest is an RNA and the protection fragment is DNA, a Nuclease Protection/ MAPS Assay (NPA-MAPS) can reduce the need for extensive handling of RNA, which can be sensitive to degradation by contaminating nucleases and thus difficult to work with. In such an NPA-MAPS assay, the probes in the probe array are oligonucleotides of the same strandedness as the nucleic acid targets of interest, rather than being complementary to them, as in a standard MAPS assay. One example of an NPA-MAPS assay is schematically represented in Figure 9.

In an NPA-MAPS assay, the target of interest can be any nucleic acid, *e.g.*,

etc.). The process of hybridization, followed by nuclease digestion and (optionally) chemical degradation, is called a nuclease protection procedure; a variety of nuclease protection procedures have been described (see, *e.g.*, Lee *et al* (1987). *Meth. Enzymol.* 152, 633-648. Zinn *et al* (1983). *Cell* 34, 865-879.). Samples treated by nuclease protection, followed by an (optional) procedure to inactivate nucleases, are placed in contact with a MAPS probe array and the usual steps of a MAPS assay are carried out. Bound protection fragments can be detected by hybridization to labeled target-specific reporters, as described herein for standard MAPS assays, or the protection fragments, themselves, can be labeled, covalently or non-covalently, with a detectable molecule.

In a preferred embodiment, the protection fragment is directly labeled, *e.g.*, rather than being labeled by hybridization to a target-specific reporter. For example, the reporter is bound to the protection fragment through a ligand-antiligand interaction, *e.g.*, a streptavidin enzyme complex is added to a biotinylated protection oligonucleotide. In another example, the protection fragment is modified chemically, (*e.g.*, by direct coupling of horseradish peroxidase (HRP) or of a fluorescent dye) and this chemical modification is detected, either with the nucleic acid portion of the protection fragment or without it, (*e.g.*, after cleavage of the modification by, for example, an enzymatic or chemical treatment). In any of the above methods, a protection fragment can be labeled before or after it has hybridized to a corresponding linker molecule.

In order to control that the nuclease protection procedure has worked properly, *i.e.* that non-hybridized nucleic acids have been digested as desired, one can design one or more protection fragments to contain overhanging (non-hybridizing) segments that should be cleaved by the nucleases if the procedure works properly. The presence or absence of the overhanging fragments can be determined by hybridization with a complementary, labeled, detection probe, or the overhanging portion of the protection fragment, itself, can be labeled, covalently or non-covalently, with a detectable molecule. This control can be performed before the sample is placed in contact with the probe array, or as a part of the MAPS assay, itself. An example of such a control assay is described in Example 15. Of course, because different labels can be easily distinguished (e.g., fluors with different absorption spectra), several differently labeled oligonucleotides can be included in a single assay. Further, the standard nuclease protection assay as analyzed by gel electrophoresis can be used during assay

signals. For example each protection fragment can be derivatized with a different natural or unnatural amino acid attached through an amide bond to the oligonucleotide strand at one or more positions along the hybridizing portion of the strand. With a mass spectrometer of appropriate energy, fragmentation occurs at the amide bonds, releasing a characteristic proportion of the amino acids. This kind of approach in which chemical moieties of moderate size (roughly 80 to 200 molecular weight) are used as mass spectrometric tags is desirable, because molecules of this size are generally easier to detect. In another example, the chemical modification is an organic molecule with a defined mass spectrometric signal, such as a tetraalkylammonium group which can, for example, derivatize another molecule such as, *e.g.*, an amino acid. In another example, positive or negative ion signals are enhanced by reaction with any of a number of agents. For example, to enhance positive ion detection, one can react a pyrylium salt (such as, *e.g.*, 2-4-dithenyl, 6-ethyl pyrylium tetrafluoroborate, or many others) with an amine to form a pyridinium salt; any of a number of other enhancing agents can be used to form other positively charged functional groups (see, *e.g.*, Quirke *et al* (1994). *Analytical Chemistry* 66, 1302-1315). Similarly, one can react any of a number of art-recognized agents to form negative ion enhancing species. The chemical modification can be detected, of course, either after having been cleaved from the nucleic acid, or while in association with the nucleic acid. By allowing each protection fragment to be identified in a distinguishable manner, it is possible to assay (*e.g.*, to screen) for a large number of different targets (*e.g.*, for 2, 6, 10, 16 or more different targets) in a single assay. Many such assays can be performed rapidly and easily. Such an assay or set of assays can be conducted, therefore, with high throughput as defined herein.

Regardless of whether oligonucleotides are detected directly by their mass or if unique molecular tags are used, the signals for each molecule to be detected can be fully characterized in pure preparations of known concentration. This will allow for the signal to be quantified (measured, quantitated) accurately. For any molecule to be detected by mass spectrometry, the intensity and profile cannot be predicted with accuracy. The tendency of the molecule to be ionized, the sensitivity of all chemical bonds within the molecule to fragmentation, the degree to which each fragment is multiply charged or singly charged, are all too complex to be predicted. However, for a given instrument with fixed energy and sample handling characteristics the intensity and profile of the signal

specific for genes that are known to be induced as part of the apoptosis (programmed cell death) process, or which are induced under conditions of cell trauma (*e.g.*, heat shock proteins) or cell toxicity (*e.g.*, p450 genes).

Other control probes can be included in an array to "fine tune" the sensitivity of an assay. For example, consider an assay for an agent which modulates the production of mRNAs associated with a particular disease state. If previous analyses have indicated that one of the correlative mRNAs (say, mRNA-A) in this set is produced in such high amounts compared to the others that its signal swamps out the other mRNAs, the linkers can be adjusted to "fine tune" the assay so as to equalize the strengths of the signals. "Blocked linkers," which comprise the anchor-specific oligonucleotide sequence designated for the mRNA-A target, but which lack the probe-specific sequence, can be added to dilute the pool of target-specific linkers and thus to reduce the sensitivity of the assay to that mRNA. The appropriate ratios of blocked and unblocked linkers can be determined with routine, conventional methods by one of skill in the art.

Samples to be tested in an assay of the invention can comprise any of the targets described above, or others. Liquid samples to be assayed can be of any volume appropriate to the size of the test region, ranging from about 100 nanoliters to about 100 microliters. In a preferred embodiment, liquid drops of about 1 microliter are applied to each well of a 1536 well microtiter dish. Samples can be placed in contact with the probe arrays by any of a variety of methods suitable for high throughput analysis, *e.g.*, by pipetting, inkjet based dispensing or by use of a replicating pin tool. Samples are incubated under conditions (*e.g.*, salt concentration, pH, temperature, time of incubation, etc.- see above) effective for achieving binding or other stable interaction of the probe and the target. These conditions are routinely determinable. After incubation, the samples can optionally be treated (*e.g.*, washed) to remove unbound target, using conditions which are determined empirically to leave specific interactions intact, but to remove non-specifically bound material. For example, samples can be washed between about one and ten times or more under the same or somewhat more stringent conditions than those used to achieve the probe/target binding.

Samples containing target RNA, *e.g.*, mRNA, rRNA, tRNA, viral RNA or total RNA, can be prepared by any of a variety of procedures. For example, *in vitro* cell cultures from which mRNA is to be extracted can be plated on the regions of a surface,

depending on the nature of the hybridized complexes and of the undesirable nucleic acids present in the sample. Reaction conditions for these enzymes are well-known in the art and can be optimized empirically. As required, the samples can be treated by well-known procedures in the art to remove unhybridized material and/or to inactivate or remove residual enzymes (*e.g.*, phenol extraction, precipitation, column filtration, etc.) . The treated samples are then placed in contact with the probe array. In order to control that specific hybridization and subsequent nuclease protection has occurred properly, one can include labeled protection fragments in the reaction mixture. In order to control that the nuclease protection procedure has worked properly, *i.e.* that non-hybridized nucleic acids has been digested as desired, one can design one or more protection fragments to contain overhanging (non-hybridizing) segments that should be cleaved by the nucleases if the assay works properly. The presence or absence of the overhanging fragments can be determined by hybridization with a complementary, labeled probe, or the overhanging portion of the protection fragment, itself, can be labeled with a detectable molecule.

For any of the methods of this invention, targets can be labeled (tagged) by any of a variety of procedures which are well-known in the art and/or which are described elsewhere herein (*e.g.*, for the detection of nuclease protection fragments). For example, the target molecules can be coupled directly or indirectly with chemical groups that provide a signal for detection, such as chemiluminescent molecules, or enzymes which catalyze the production of chemiluminsecent molecules, or fluorescent molecules like fluorescein or cy5, or a time resolved fluorescent molecule like one of the chelated lanthanide metals, or a radioactive compound. Alternatively, the targets can be labeled after they have reacted with the probe by one or more target-specific reporters (*e.g.*, antibodies, oligonucleotides as shown in Fig. 1, or any of the general types of molecules discussed above in conjunction with probes and targets). A variety of more complex sandwich-type detection procedures can also be employed. For example, a target can be hybridized to a bifunctional molecule containing a first moiety which is specific for the target and a second moiety which can be recognized by a common (*i.e.*, the same) reporter reagent, *e.g.*, a labeled polynucleotide, antibody or the like. The bifunctional molecules can be designed so that any desired number of common reporters can be used in each assay.

Methods by which targets can be incubated with a target-specific reporter(s)

or other detection label); and the ratio of the two signals can be determined. The presence of control linkers permits calibration of the number of functional (*e.g.*, able to interact with linkers) anchors within and between test regions (*i.e.* tests the capacity of each locus of the array to bind target, for purposes of normalizing signals), serves as a basis for quantitation of the amount of bound target, aids in localization of the anchor loci and/or provides a positive control, *e.g.*, in cases in which there is no signal as a result of absence of target in a sample. In one embodiment of the invention, two different labels (*e.g.*, fluorophores) can also be used to detect two different populations of target molecules; however, the ability to recognize the presence of targets by spatial resolution of signals allows the use of a single type of label for different target molecules.

In another embodiment of the invention, "anchors" which are specific for a target(s) of interest are not associated with linkers, but rather are associated directly with the target(s); the target(s), in turn, can interact optionally with a target-specific reporter(s). Targets, whether labeled or unlabeled, can be detected by any of a variety of procedures, which are routine and conventional in the art (see, *e.g.*, Fodor *et al* (1996). U.S. Pat. No. 5,510,270; Pirrung *et al* (1992). U.S. Pat. No. 5,143,854; Koster (1997). U.S.Pat. No. 5,605,798; Hollis *et al* (1997) U.S. Pat. No. 5,653,939; Heller (1996). U.S.Pat. No. 5,565,322; Eggers *et al* (1997). U.S.Pat. No. 5,670,322; Lipshutz *et al* (1995). *BioTechniques* 19, 442-447; Southern (1996). *Trends in Genetics* 12, 110-115). Detection methods include enzyme-based detection, colorimetric methods, SPA, autoradiography, mass spectrometry, electrical methods, detection of absorbance or luminescence (including chemiluminescence or electroluminescence), and detection of light scatter from, *e.g.*, microscopic particles used as tags. Also, fluorescent labels can be detected, *e.g.*, by imaging with a charge-coupled device (CCD) or fluorescence microscopy (e.g., scanning or confocal fluorescence microscopy), or by coupling a scanning system with a CCD array or photomultiplier tube,or by using array-based technology for detection (*e.g.*, surface potential of each 10-micron part of a test region can be detected or surface plasmon resonance can be used if resolution can be made high enough.) Alternatively, an array can contain a label (*e.g.*, one of a pair of energy transfer probes, such as fluorescein and rhodamine) which can be detected by energy transfer to (or modulation by) the label on a linker, target or reporter. Among the host of fluorescence-based detection systems are fluorescence intensity, fluorescence

Rec: Receptor protein. Ligand: a natural or synthetic ligand for the receptor. *: a fluorescent labeling molecule attached to the Ligand.

Fig. **4** illustrates a surface which comprises 21 test regions, each of which is further subdivided into 16 subregions (indentations, dimples).

Figs. **5a, 5b** and **5c** illustrate three pieces from which a surface such as that shown in Fig. 4 can be assembled. Fig. 5a represents a well separator; Fig. 5b represents a subdivider; and Fig. 5c represents a base.

Fig. **6** represents two test regions, each of which comprises a linear array of probes (or anchors) which are in a "bar-code"- like formation.

Fig. **7** schematically represents a test region comprising 3 anchors (A, B and C), each of which is present in multiple copies (a "group"). The location of each group of anchors is termed a "locus."

Fig. **8** illustrates an assay in which cDNA(s) generated by specific reverse transcriptase are assayed on MAPS plates.

Fig. **9** illustrates an assay which uses a nuclease protection procedure (NPA-MAPS assay). Sample RNA is prepared from cells or from tissue and is represented as thin wavy lines. To the RNA sample is added a group of polynucleotide protection fragments, portrayed as thick, dark and light lines. The dark sections of the protection fragments represent segments that are complementary to specific RNA targets and hybridize to those targets. The light sections represent overhanging portions: sequences contiguous with the complementary sequence but not complementary to target. The protection fragments are added in excess. Following hybridization of all available target to the protection fragments, the samples are treated with an appropriate cocktail of nucleases and with chemical treatments that destroy unwanted non-hybridized RNA and non-hybridized polynucleotide. For example, S1 nuclease can destroy any single stranded DNA present. Hence, excess protection fragment is hydrolyzed as is the overhanging non-hybridized portion of bound protection fragment. RNA can be hydrolyzed by addition of ribonucleases including ribonuclease H and or by heating samples in base. Remaining is a collection of cleaved protection fragments that reflect how much of each target RNA had been present in the sample. The remaining protection fragments are measured by a MAPS hybridization assay.

Fig. **10** illustrates hybridization specificity in a MAPS assay.

conditions of stringency, the 16 wells of the microplate are imaged with a CCD-based fluorescence imager, for example. Fig. 20a shows stylized results. It is expected that each EST-specific detector oligonucleotide should label the mRNA or cDNA held down by the corresponding EST-specific probe. For example, probe 5 assembles the cDNA or mRNA containing the fifth EST sequence at that locus, so the fifth detector oligonucleotide should also hybridize to the cDNA or mRNA at the same locus. This is the case for these stylized data, with each detection oligonucleotide labeling the matching probe. In addition, the first three detector oligonucleotides each label cDNA or mRNA held down by the first three probes, showing that these sequences lie along the same gene. Similarly, the last five ESTs appear to be linked. The linkage assigned from these data are presented graphically in Fig. 20b.

Fig. **21** illustrates the relationships of the probes, detector oligonucleotides and ESTs #1, 2 and 6 shown in Figures 18-20.

Fig. **22** illustrates a high throughput assay.

EXAMPLES

Example 1 __**Hybridization Specificity**__ **(see Figure 10)**

A generic MAPS plate was produced by using an inkjet dispenser, the Pixus system (Cartesian Technologies, Inc., Irvine, CA) to form an identical grid of DNA within each well of a microtiter plate. All oligonucleotides were purchased from Biosource International (Camarillo, CA). For this plate, seven different oligonucleotide anchors were dispensed within each well in the pattern shown as the Key (left side of the figure). Each oligonucleotide was dispensed as a 10 nanoliter droplet to two spots, from a 2 uM solution containing 500 mM sodium phosphate pH 8.5 and 1 mM EDTA to the wells of a DNA Bind plate (Corning Costar), and allowed to dry. After attachment, wells were blocked with 50 mM Tris pH 8, and then oligonucleotide that had not covalently attached to the surface was washed away with 0.1% SDS in 5x SSP buffer.

To the washed plate fluorescently labeled linker oligonucleotides were added and allowed to hybridize in 6x SSPE with 0.1% Triton X-100 at room temperature for thirty minutes. This is a preferred protocol for attachment of linkers. The linker oligonucleotides were cy5 [illegible]vatized during synthesis, and were complementary in 25 base-pair segments to specific anchoring oligonucleotides. The sequences of the seven

CTGGCAGCCACGGACGCGGAACGAG

#6 Anchor*: SEQ ID:19

CGGTCGGCATGGTACCACAGTCCGC

Linker** SEQ ID:20

GCGGACTGTGGTACCATGCCGACCG

#7 Anchor*: SEQ ID:21

GCGCGCCGCGTTATGCATCTCTTCG

Linker** SEQ ID:22

CGAAGAGATGCATAACGCGGCGCCG

*Anchors were synthesized with C12 spacer with amide at the 5' end
**Linkers were synthesized with Cy5 attached at the 5' end
***Detector Oligonucleotides were synthesized with biotin attached at the 5' end

To each well either one linker or a mixture of linkers (as indicated in the figure) was added in bulk. (To the well marked "all" was added a mixture of all seven linkers.) Following incubation and washing in 5x SSP 3 times, the fluorescence picture shown on the right portion of the figure was taken with a Tundra imager (IRI, St. Catherines, Ontario). As can be seen, the linkers self-assembled to the surface, by specifically associating with their complementary anchors.

This process is repeated except that eight different anchors are dispersed in each well and linkers subsequently preferentially associated therewith. The entire process is repeated with 36, 64 etc. different anchors in each well of a 24, 96, 384, 864 or 1536 well plate.

Example 2 **Binding Kinetics** **(see Figure 11)**

The rate of hybridization of Cy5-derivatized linker number 1 to its complementary attached anchor is shown, for different concentrations of linker. The generic MAPS plate was prepared as for figure 1, except anchor 1 was attached at four spots per well.Incubations were done at room temperature in 5x SSP with 0.1% tween-20, wells were washed 3 times with 5x SSP, and bound fluorescence was measured. A fluorescence picture of the plate was taken with the Tundra, and background was subtracted and the integrated intensity of each spot within each well was calculated with Tundra software. Plotted is the average and standard deviation for the integrated intensity for the four spots within each of two duplicate wells.

Example 5 **Assay of Two Oligonucleotides** **(see Figure 12)**

A binding curve demonstrating a MAPS hybridization assay using the preferred protocol discussed above for two different target oligonucleotides is shown. A generic MAPS plate was prepared with four different anchoring oligonucleotides each spotted four times within each well. For the second and fourth anchor, complementary linker oligonucleotides were self-assembled onto the surface as described. Two targets were added at the concentrations shown in 40 microliters to each well as described, and incubated at 50° C overnight. The amount of each target attached was visualized by attaching biotinylated detection oligonucleotide specific for each target followed by HRP:SA and chemiluminescence imaging as described. In the lower panel the intensity of the image is quantified. Software that is part of the Tundra Imager package was used to scan the intensity of the images along lines between the arrows shown in the upper panel. At the lowest concentration of target, 1.1 pM, the scanned images show well-defined gaussian peaks at each spot, while there are no discernable background peaks seen in the left-most sample, at 0 concentration of target.

Example 6 **Sensitivity Shifting** **(see Figure 13)**

A MAPS hybridization assay can be used for measuring the concentration of a set of oligonucleotides, by binding them to a surface and labeling them. This works well for those oligonucleotides which are at modest or low concentration. Two samples can be distinguished in such a case because if one sample contains more oligonucleotide, more will bind. On the other hand, if the concentration of targeted oligonucleotide is saturating for the surface (*i.e.* if it is high enough to occupy all binding sites), then if the concentration goes up no more can bind, so the amount cannot be measured. However, the binding curve of a target can be shifted by adding unlabeled competing ligand.

Binding data are obtained for four different oligonucleotide targets, all of which saturate the surface (*i.e.* reach maximal binding) at roughly 3 nM. By adding unlabeled competitive targets to all wells, the binding of labeled oligonucleotide is shifted, so that less binds at the lower concentration, and the level at which saturation occurs is moved up. One can add competitive oligonucleotides for, say, targets 1 and 3 but not 2 and 4. This shifts the sensitivity of the assay only for targets 1 and 3. In this way oligonucleotide targets of widely different concentrations can be measured within one

Then either alkaline phosphatase coupled to streptavidin (AlkPhos:SA) or HRP:SA is added, followed by washing and addition of either CDP-Star (Tropix) to the wells with AlkPhos:SA or ECL-Plus to the wells with HRP:SA. Labeling with SA derivatized enzymes and substrates is as suggested by the manufacturers for use in labeling of western blots. These two (as well as other available substrates) can both be used to assess oligonucleotide hybridization to MAPS plates.

Example 10 Resolution at 0.6 mm.

The resolution of the current system for MAPS assay is tested by preparing a MAPS plate with four different oligonucleotide anchors per well each spotted four times per well, with a pitch (center-to-center spacing) of 0.6 mm. Then either cy5-derivatized linkers or biotinylated linkers are hybridized and detected and scanned as above. For the epi-fluorescence measurement the resolution is higher (and pitch could likely be reduced). For the chemiluminescence detection procedure neighboring spots are not completely separated, yet at this spacing individual peaks may be resolved unambiguously by computer deconvolution.

Example 11 Test Nuclease Protection Protocol.

In an assay to test for the optimal conditions for hybridization and nuclease treatment for the nuclease protection protocol, the Nuclease Protection Assay kit from Ambion (Austin, Texas) is used to provide conditions, buffers and enzymes. Eight samples are prepared in one of three buffers. Hyb Buff 1 is 100% Hybridization Buffer (Ambion); Hyb Buff 2 is 75% Hybridization Buffer and 25% Hybridization Dilution Buffer (Ambion); and Hyb Buff 3 is 50% of each. A 70-mer oligonucleotide that contains 60 residues complementary to a test mRNA is synthesized (Biosource International, Camarillo, CA) and labeled with Psoralen-fluorescein (Schleicher and Schuell, Keene, NH) following the protocol as suggested for labeling of Psoralen-biotin by Ambion. Briefly, protection fragment is diluted to 50 ug/ml in 20 μls of TE buffer(10 mM Tris, 1 mM EDTA, pH 8) boiled for 10 minutes, and rapidly cooled in ice water. Four μls of 130 ug/ml Psoralen-fluorescein in DMF is added, and the sample is illuminated for 45 minutes at 40° C with a hand-held long wavelength UV source. Free Psoralen-fluorescein is removed by extraction with saturated butanol. The mRNA used

5 minutes, and allowed to cool to 19° C and incubated for 19 hours. 200 μls of nuclease mixture was then added to each sample for 30 minutes at 19° C. 60 μls was aliquoted from each sample for the MAPS assay. 2 μl of 10 N NaOH and 2 μl of 0.5 M EDTA was added, and the sample heated to 90° C for 15 minutes, 37° C for 15 minutes, and allowed to sit at room temperature for 20 minutes. Then samples were neutralized with 2 μl of 10 M HCl, and 12 μls of 20x SSC containing 2 M HEPES pH 7.5 and 200 nM biotinylated detector oligonucleotide specific for the protection fragment was added along with 1 μl of 10% SDS. Samples were mixed, heated to 80° C for 5 minutes, and two 35 μl aliquots of each sample were pipetted to two wells of a MAPS plate (each sample was split in two and run in duplicate on the MAPS plate). The plate had been prepared as for standard MAPS protocol, with self-assembled CY5-derivatized linker specific for the protection fragment already attached. The MAPS plate was covered and incubated at 50° C overnight, and detection and luminescence performed as described. In the last sample, no nucleases were added during the assay as a control to visualize how the protection fragment alone would be detected by MAPS. In the lower portion of the figure, the intensity scan (as analyzed by the imager) for the top row of wells is presented. The amount of GAPDH mRNA present in the sample (that is, the amount in each duplicate well after aliquoting to the MAPS plate) is listed in the figure.

The oligonucleotides used for the MAPS plates were as follows:

Anchor*: SEQ ID: 25
CGCCGGTCGAGCGTTGTGGGAGCGC
Linker** SEQ ID: 26
CTTGAGTGAGTTGTCATATTTCTCGGATACTGAGTGCGCTCCCACAACGCTCGACC
GGCG
Protection fragment (complementary to mouse antisense mRNA for GAPDH)
SEQ ID: 27
CGAGAAATATGACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAAC
TGCTTGCTTGTCTAA
Detector Oligonucloeotide*** – labeled at 5' end with biotin SEQ ID: 28
AAGCAGTTGGTGGTGCAGGATGCAT

*Anchors were synthesized with C12 spacer with amide at the 5' end
**Linkers were synthesized with Cy5 attached at the 5' end
***Detector Oligonucleotides were synthesized with biotin attached at the 5' end

(samples include 500, 170, 50, 5, or 0.5 μg of total mouse RNA. Two control samples are included to which no S1 nuclease is added. Signal is seen only for the complementary protection fragment.

Oligonucleotides used:

For Antisense Control (same oligonucleotides as for example 12):

Anchor*: SEQ ID: 25
CGCCGGTCGAGCGTTGTGGGAGCGC

Linker** SEQ ID: 26
CTTGAGTGAGTTGTCATATTTCTCGGATACTGAGTGCGCTCCCACAACGCTCGACC GGCG

Protection fragment (complementary to mouse antisense mRNA for GAPDH) SEQ ID: 27
CGAGAAATATGACAACTCACTCAAGATTGTCAGCAATGCATCCTGCACCACCAAC TGCTTGCTTGTCTAA

Detector Oligonucloeotide*** SEQ ID: 28
AAGCAGTTGGTGGTGCAGGATGCAT

For Sense GAPDH mRNA samples:

Anchor*: SEQ ID: 25
CGCCGGTCGAGCGTTGTGGGAGCGC

Linker** SEQ ID: 29
ATGCATCCTGCACCACCAACTGCTTGATACTGAGTGCGCTCCCACAACGCTCGAC CGGCG

Protection fragment (complementary to mouse mRNA for GAPDH): SEQ ID: 30
AAGCAGTTGGTGGTGCAGGATGCATTGCTGACAATCTTGAGTGAGTTGTCATATT TCTCGGCTTGTCTAA

Detector Oligonucleotide*** SEQ ID: 31
CGAGAAATATGACAACTCACTCAAG

*Anchors were synthesized with C12 spacer with amide at the 5' end
**Linkers were synthesized with Cy5 attached at the 5' end
***Probes were synthesized with biotin attached at the 5' end

Example 15 <u>A Nuclease Protection MAPS Assay with Controls.</u>

mRNA isextracted from mouse liver and nuclease protection is performed essentially as described in Example 14, except that the GADPH specific protection fragment comprises 60 nucleotides which are complementary to mouse GAPDH, followed by 15 "overhanging" nucleotides at the 3' end of the fragment which are not complementary to the target. After hybridization and nuclease digestion the remaining protection fragment is hybridized to a MAPS plate as indicated in Example 14, except that two different oligonucleotide detection fragments are used to detect the immobilized

development of drugs for treating this kind of tumor.

About 10,000 to 100,000 cells are added to each well of 100 96-well polystyrene plates and the cells are grown for 2 days until they cover the surface of each well. For 8 wells of each plate, the cells are left to grow without additions. To the remaining 88 wells of each plate, a different chemical compound is added so that the effect of it alone can be tested. For the 100 plates used at one time, 8800 compounds can be tested or screened. The cells are grown for 24 hours in the presence of the compounds, and then the cells are harvested for assay. The cells in each plate are treated according to the instructions for preparing RNA in samples from 96-well plates (for example according to the Qiagen RNeasy 96 kit). After the RNA is prepared, the amount of each of 36 different mRNA species is quantified by the NPA-MAPS approach, including the 30 correlative genes and 6 normal "housekeeping" genes. 36 DNA oligonucleotide protection fragments, each corresponding to one of the genes of interest, are added to each well and allowed to hybridize under selected stringent conditions to their target mRNA sequences. Then S1 nuclease is added to destroy excess unhybridized DNA, and the samples are treated chemically to destroy the RNA as well. Left is the oligonucleotide protection fragment for each of the 36 genes in proportion to how much mRNA had been present in the treated cells for each sample.

One hundred 96-well plates, each of which comprises an array of a plurality of 36 different anchor oligonucleotides in each well, are prepared by adding to each well 36 different linker oligonucleotides. The linkers self-assemble on the surface of each well, converting the generic plates to MAPS plates comprising specific probes for each of the 36 oligonucleotide protection fragments. Each linker has a portion specific for one of the 36 anchors and a portion specific for a segment of one of the 36 protection oligonucleotides. The oligonucleotide sample from each well of the 100 sample plates is added to a corresponding well of the 100 MAPS plates. After hybridization under selected stringent conditions, a detection oligonucleotide for each target with a chemiluminescent enzyme attached is added, so that each specific spot of each well lights up in proportion to how much mRNA had been present in the sample. Any wells that show reduced amounts of correlative genes with no effect on the 6 house keeping genes are interesting. The compounds added to the cells for those samples are possible starting points to develop anti-tumor agents.

array (with anchors only) to arrays comprising peptide substrates. The 36 peptide substrates are synthesized and each is attached covalently through an amide bond, for example, to an oligonucleotide containing a 5' amino group. The oligonucleotides contain sequences that hybridize specifically to the anchors. The peptide/oligo linkers are self assembled on the surface by adding them to all wells of the MAPS plates.

For screening, the five kinases at appropriate concentrations (so that the rates of phosphorylation of the substrates are balanced as much as possible) are added to each well along with one of 8800 different compounds to be tested. The compounds are tested for their ability to directly inhibit the isolated enzymes. The amount of phosphorylation of each arrayed peptide is detected by adding labeled antibodies that bind only to peptides that are phosphorylated on tyrosine. Any wells that show a reduction in some of the phospho-tyrosine spots but not all of the spots are interesting. Compounds that had been added to those wells can be tested further as possible selective inhibitors of some of thekinases tested.

The scheme of the assay is shown in the top panel of Figure 17. A chimeric linker molecule is prepared in which a 25 base pair oligonucleotide complementary to one of the anchors is crosslinked to a peptide substrate of a tyrosine phosphokinase enzyme. The chimeric oligo-peptide substrate self-assembles onto an array of oligonucleotide anchors, the kinase enzyme is used to phosphorylate the peptide portion of the chimera, and after the enzyme reaction is allowed to proceed, the amount of phosphorylation of the peptide is determined by anti-phoshotyrosine or anti-phosphoserine antibodies with an attached detection fluorophore or enzyme.

The results of the assay are shown in the lower panel. The homobifunctional crosslinker, DSS (Pierce), was used to attach the 5' amino group of an oligonucleotide linker to the N terminus of a peptide synthesized with a phosphorylated tyrosine. The sequence of the peptide in single-letter code was: TSEPQpYQPGENL (SEQ ID: 32), where pY represents phosphotyrosine. The chimera was either used directly or first brought to pH 14 for 60 minutes in order to partially hydrolyze the phosphate group from the tyrosine. The phosphorylated or partially dephosphorylated chimeric molecules were self-assembled onto complementary anchor molecules within a MAPS plate at the concentrations shown for one hour. After washing and blocking the wells with 0.3% BSA in SSPTP antiphosphotyrosine antibody crosslinked to HRP (antibody 4G10 from

hybridization assays was very high. The left-most and right-most columns served as controls to standardize the signal for different concentrations of the oligonucleotide.

In a similar fashion, 16 different oligonucleotides can be tested in each well, and the test repeated in the 80 different wells of the plate. Of course, an even greater number of different oligonucleotides or other probes, (*e.g.*, 100 nucleotide probes) can be assayed in each well, and many plates can be tested simultaneously (*e.g.*, 100 plates, such as 96-well microtiter plates). The large number of assays which can be performed on each sample (*e.g.*, in the latter case, about 100 different assays) and the large number of samples which can be assayed simultaneously (*e.g.*, in the latter case, about 96 x 100, or 9600 different samples) provides for very high throughput.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make changes and modifications of the invention to adapt it to various usage and conditions.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The preceding preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever.

The entire disclosure of all applications, patents and publications, cited above and in the figures are hereby incorporated by reference.

and a second portion that comprises an oligonucleotide probe which corresponds to at least one of said ESTs.

2. A method of determining which of a plurality of ESTs are complementary to a given nucleic acid, comprising,

a) incubating an immobilized array of oligonucleotide probes, at least one of which corresponds to each of said ESTs, with a test sample which may contain said given nucleic acid, to obtain a hybridization product between said oligonucleotide probes and said nucleic acid,

b) incubating said hybridization product with a detector oligonucleotide, which corresponds to an EST to which one of said oligonucleotide probes corresponds, but which is specific for a different portion of the EST than is said oligonucleotide probe, and

c) detecting which oligonucleotide probes of said array are labeled by said detector oligonucleotide,

wherein said array of oligonucleotide probes is immobilized on a region of a combination, wherein said combination comprises

1) a surface comprising a number of spatially discrete, substantially identical, regions equal to the number of ESTs to be studied, each region comprising

2) a number of different anchors equal to the number of ESTs to be studied, each anchor in association with

3) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises an oligonucleotide probe which is corresponds to at least one of said ESTs.

3. The method of claim 1, further comprising, after a), b) and/or c), removing unbound portions of said sample.

4. The method of claim 2, further comprising, after a) and/or b), removing unbound portions of said sample.

5. A method of determining which of a plurality of ESTs are complementary to a given nucleic acid, comprising,

a) incubating a collection of bifunctional oligonucleotide linker molecules, each of which comprises a first portion which is a probe that corresponds to at least one of said ESTs, and a second portion which is specific for an anchor oligonucleotide, with

detector oligonucleotide which corresponds to a polynucleotide to which a given one of the oligonucleotide probes of said array corresponds, thereby binding detector oligonucleotides to nucleic acid molecules which have bound to said given oligonucleotide probe or to other oligonucleotide probes which are complementary to said nucleic acid,

d) detecting the presence of said detector oligonucleotides, thereby identifying which polynucleotide-corresponding oligonucleotide probes of said array are complementary to portions of a nucleic acid which binds to said given oligonucleotide polynucleotide-corresponding probe, thereby identifying which polynucleotides are complementary to said given nucleic acid,

wherein said array of oligonucleotide probes is immobilized on a region of a combination, wherein said combination comprises

1) a surface comprising a number of spatially discrete, substantially identical, regions equal to the number of polynucleotides to be studied, each region comprising

2) a number of different anchors equal to the number of polynucleotides to be studied, each anchor in association with

3) a bifunctional linker which has a first portion that is specific for the anchor, and a second portion that comprises an oligonucleotide probe which corresponds to at least one of said polynucleotides.

7. The method of claim 5, further comprising, after a), b) and/or c), removing unbound portions of said sample.

8. The method of claim 6, further comprising, after a), b) and/or c), removing unbound portions of said sample.

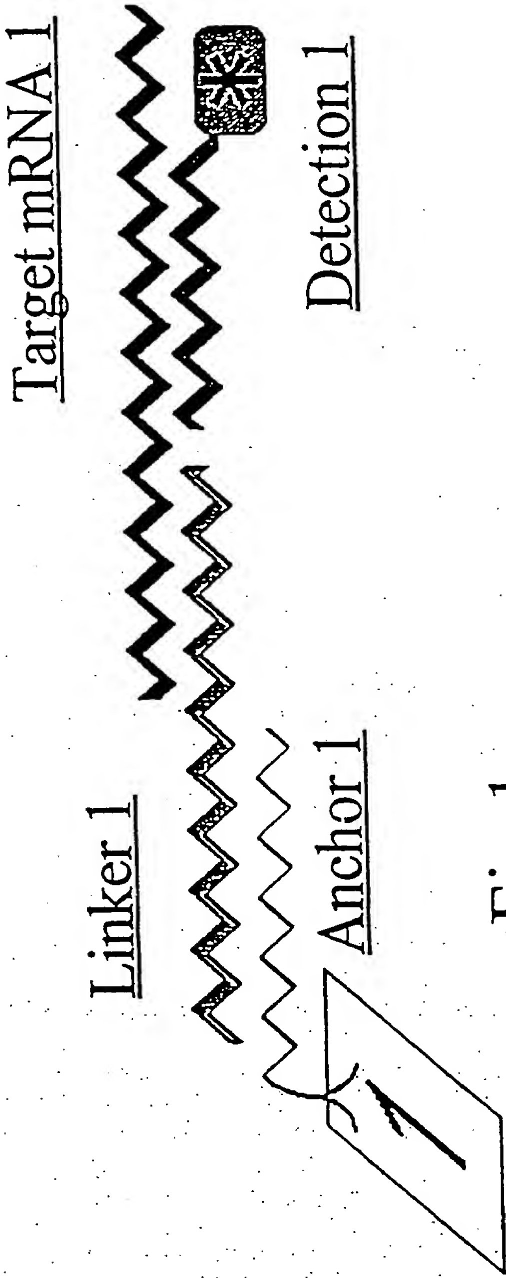

Fig 1

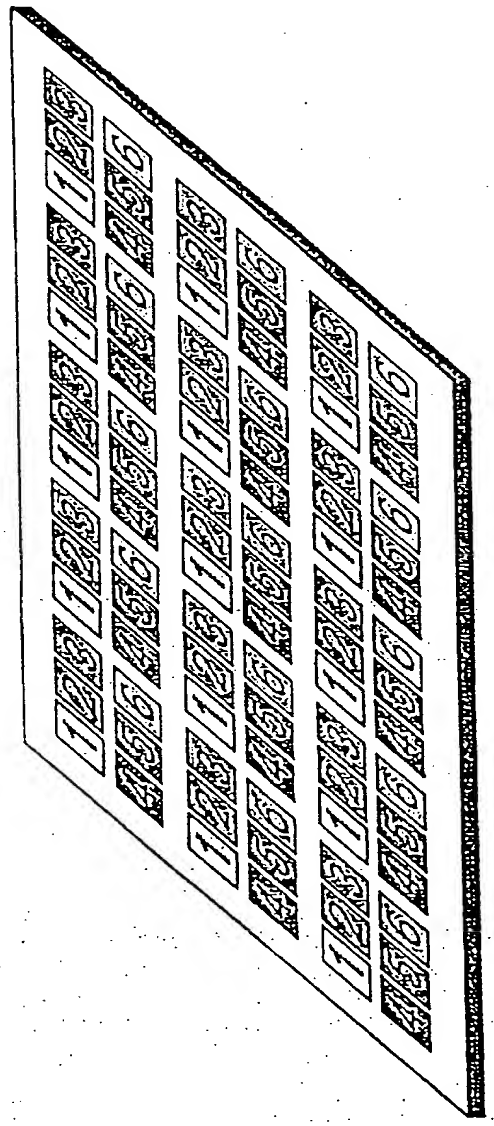

Fig 2

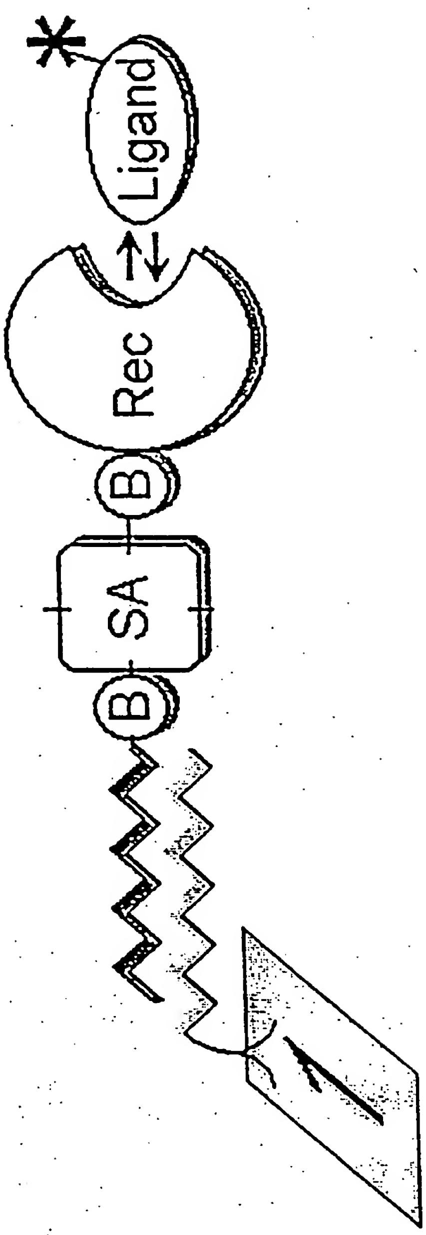

Fig 3

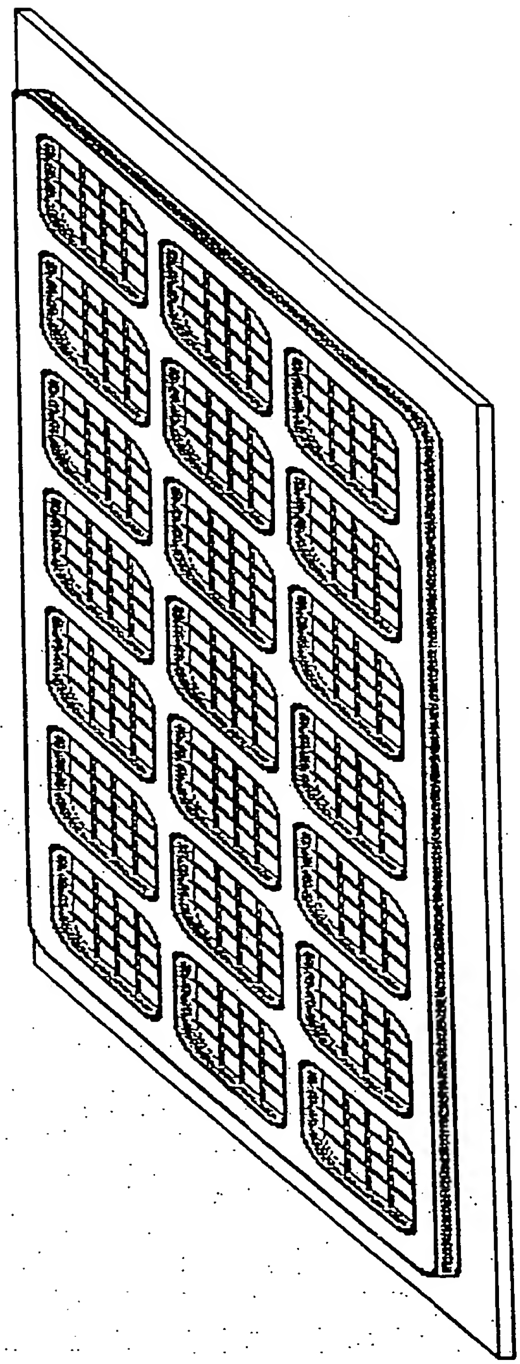

Fig 4

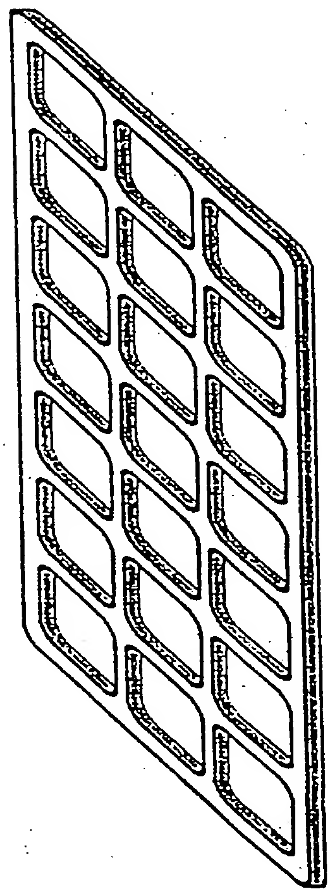

Fig 5a

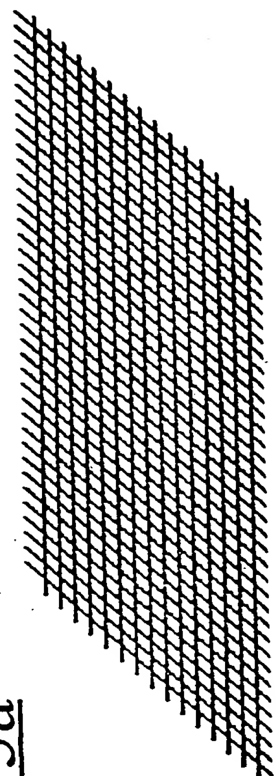

Fig 5b

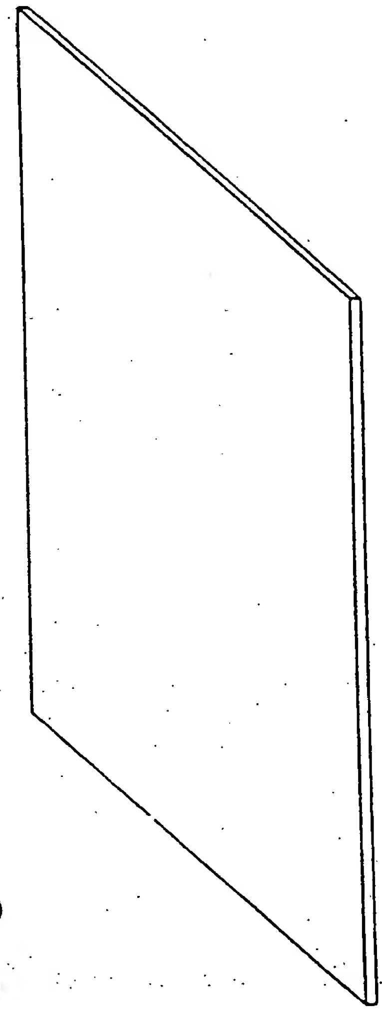

Fig 5c

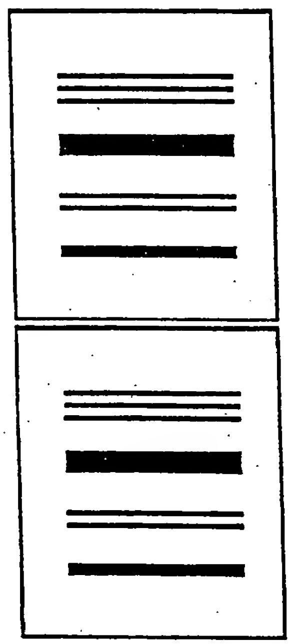

Fig 6

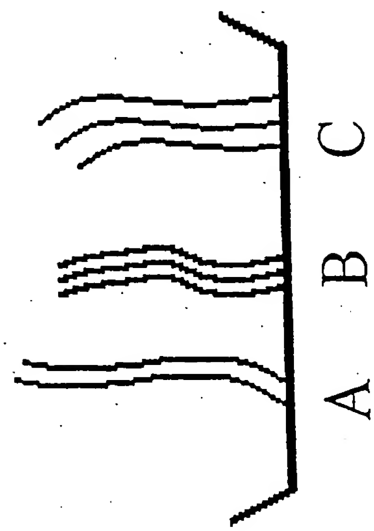

Fig 7

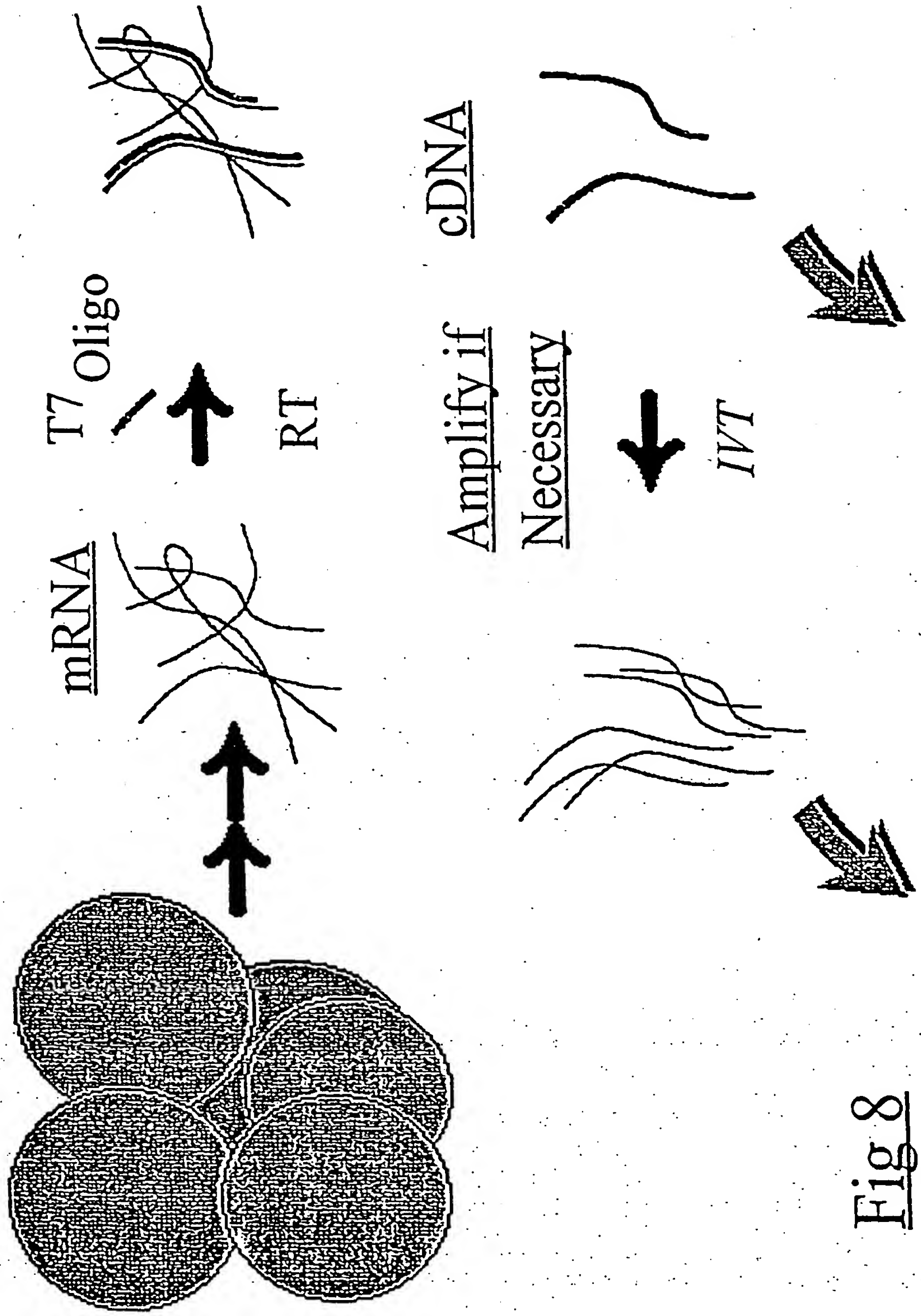

Fig 8

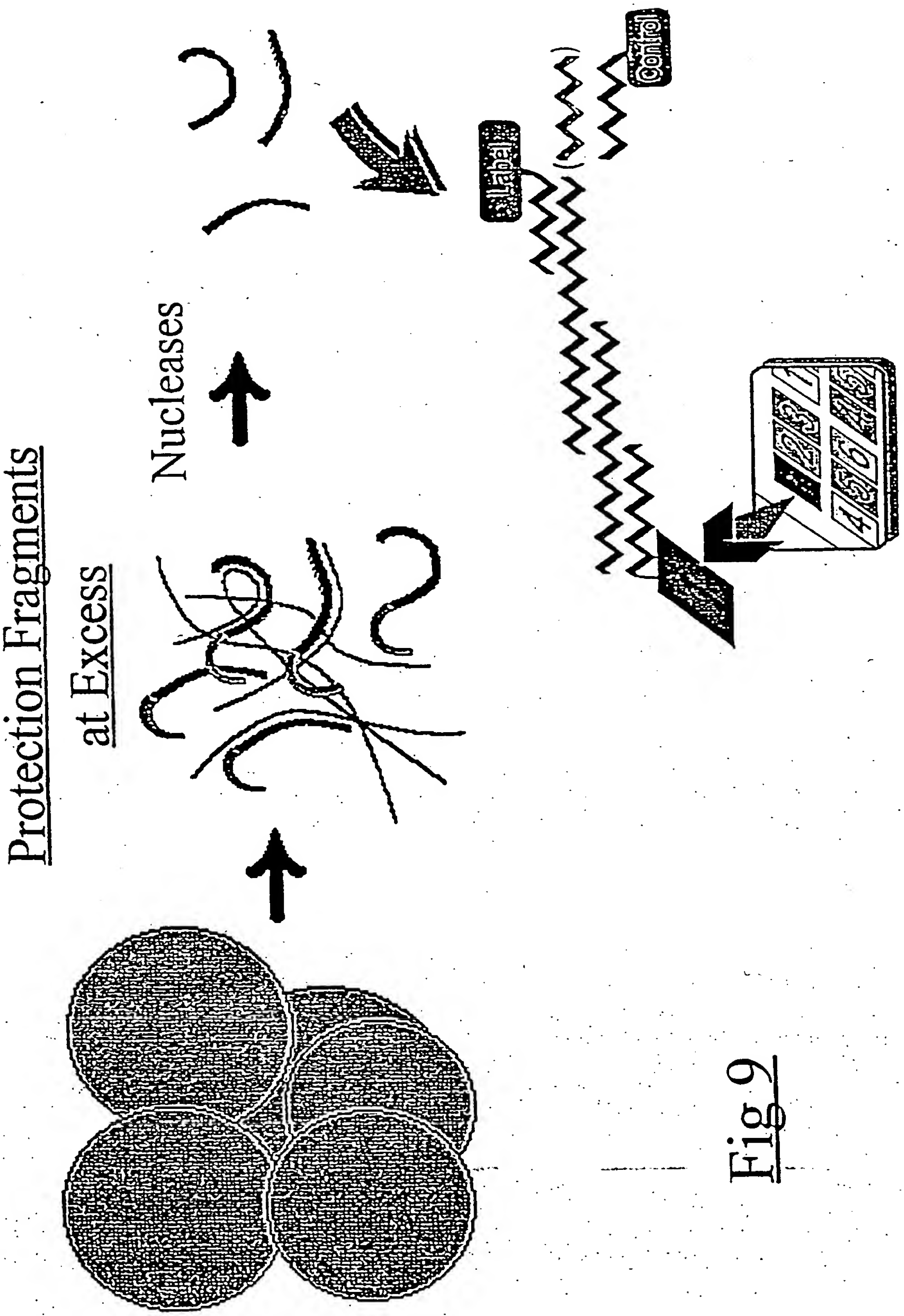

Fig 9

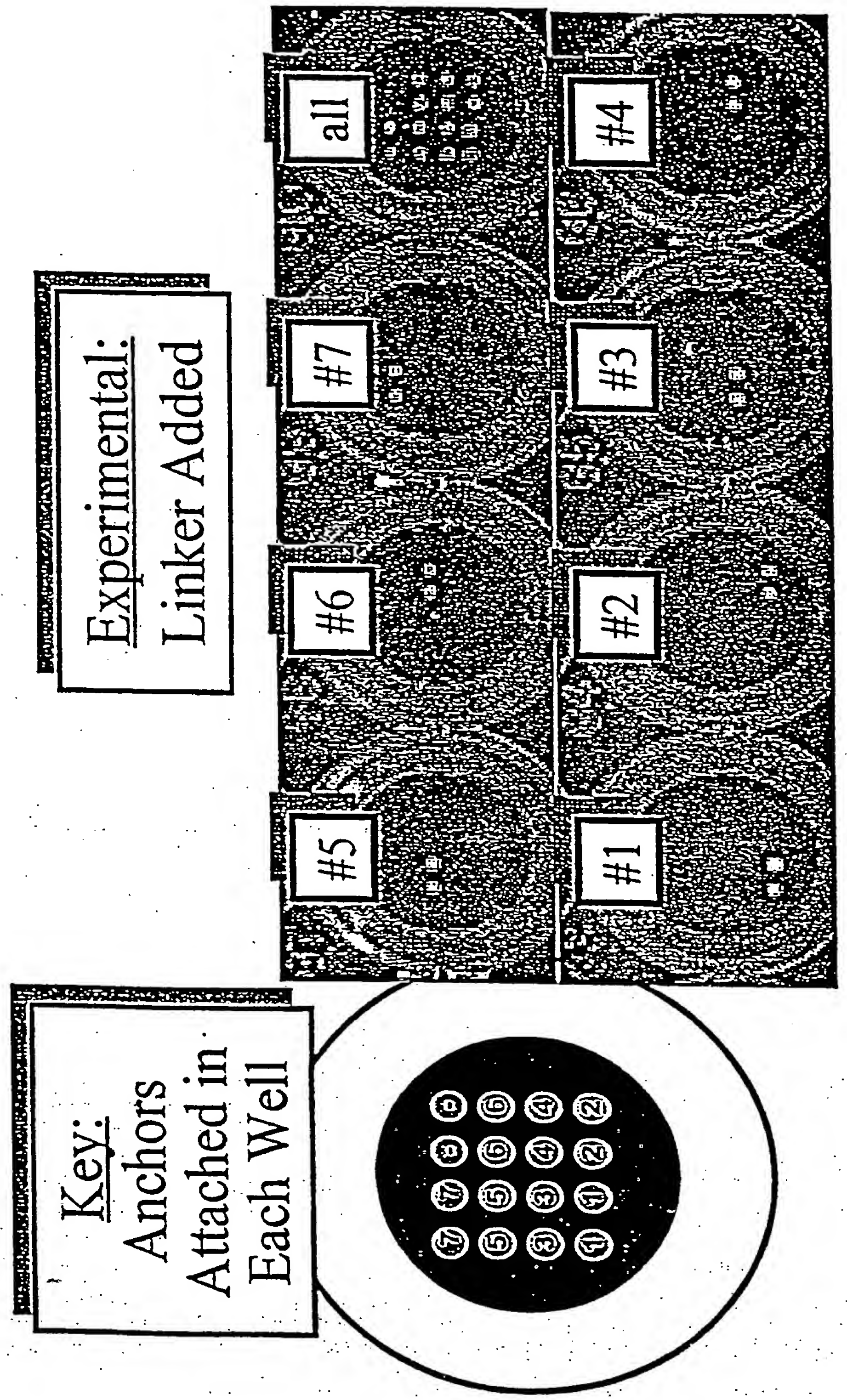
Experimental:
Linker Added
all
#7
#6
#5
#4
#3
#2
#1
Key:
Anchors
Attached in
Each Well

Fig 10

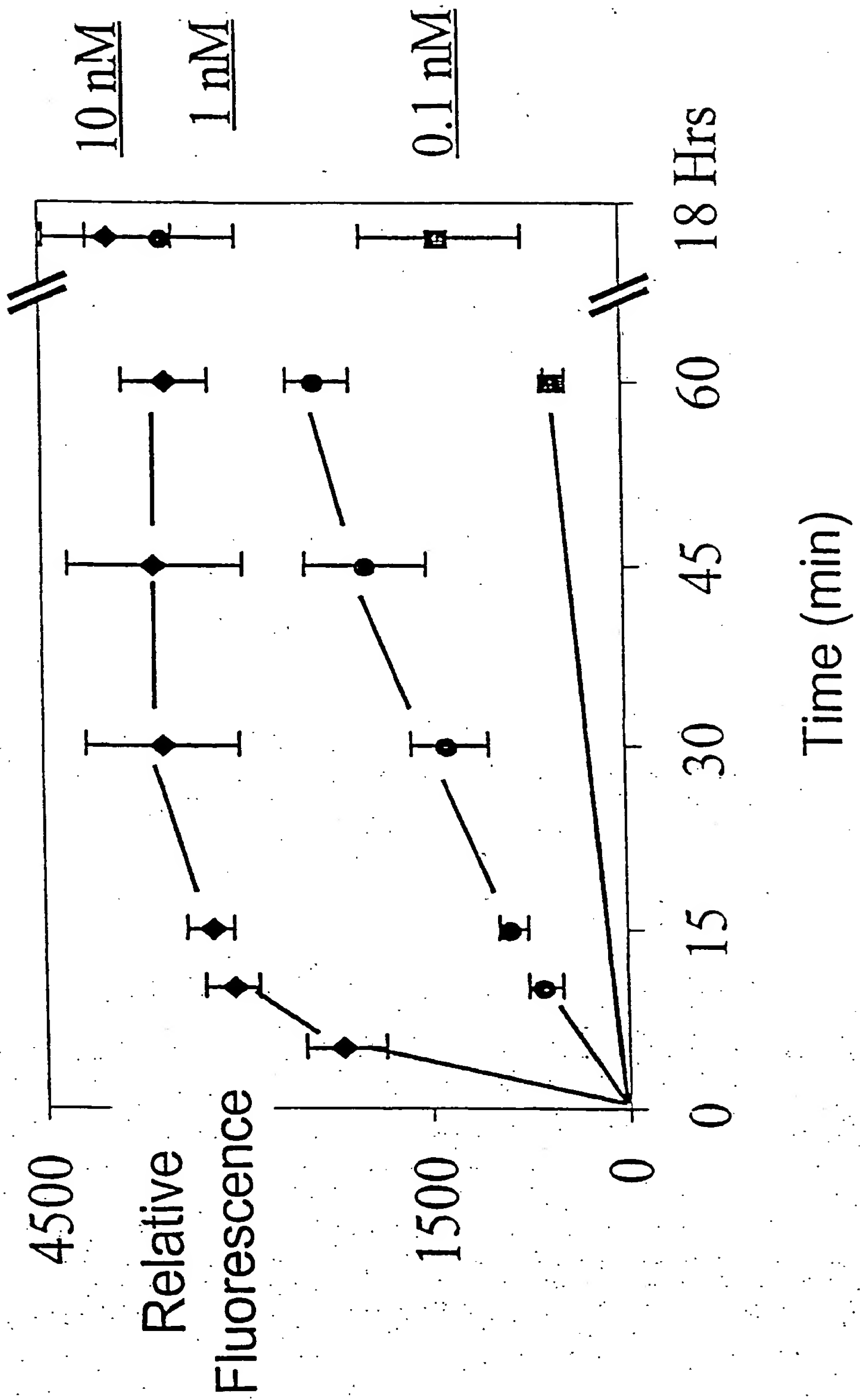
10 nM
1 nM
0.1 nM
Relative Fluorescence
4500
1500
0
0
15
30
45
60
18 Hrs
Time (min)

Fig 11

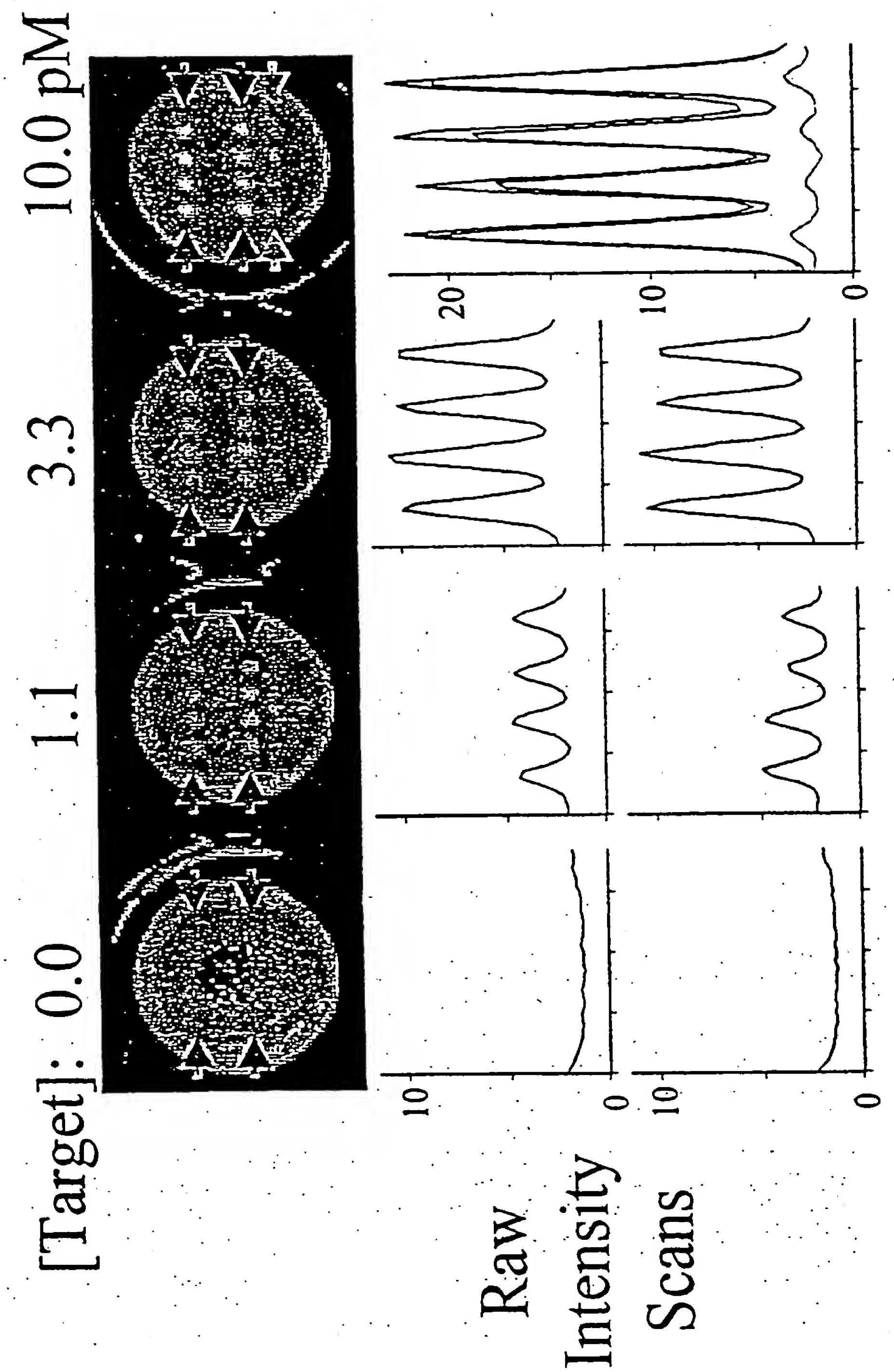

Fig 12

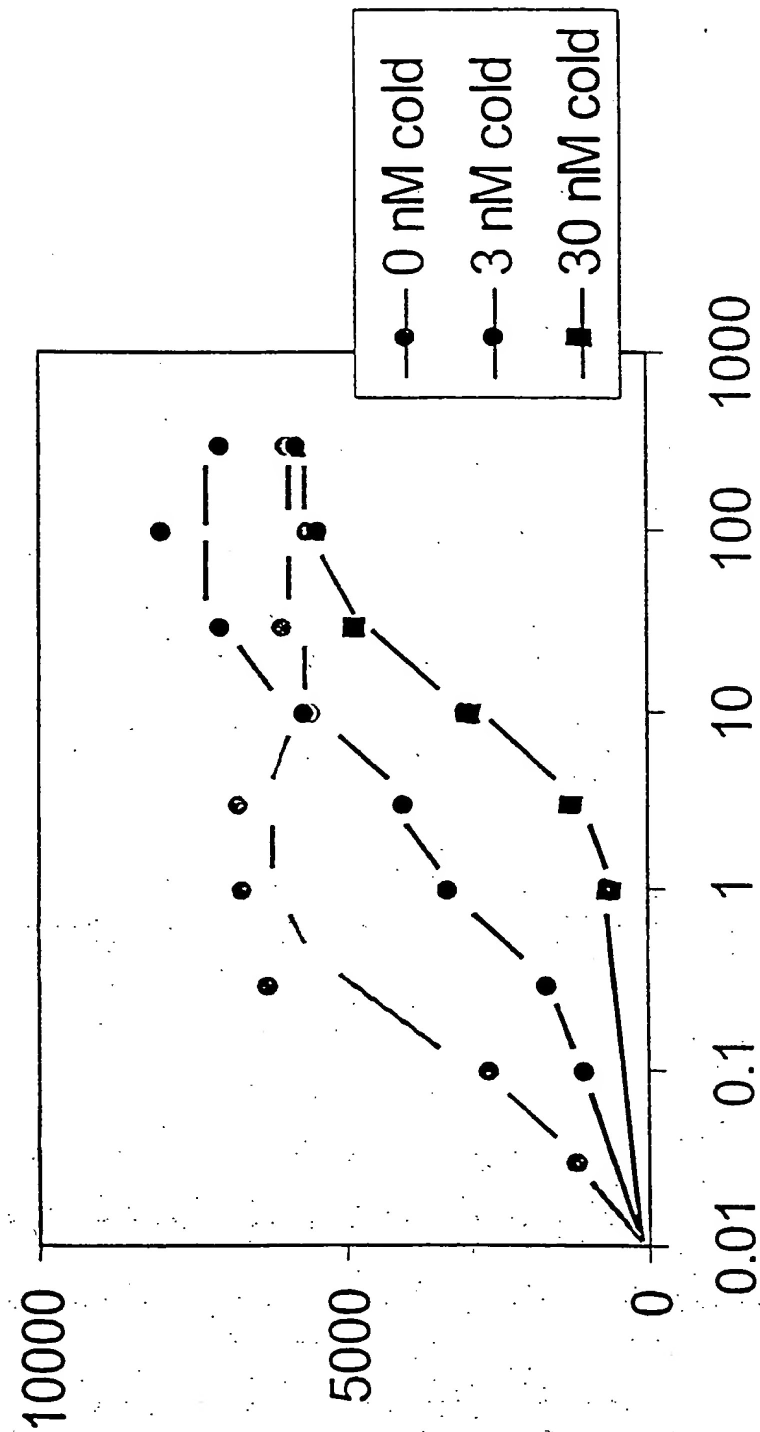

Fig 13

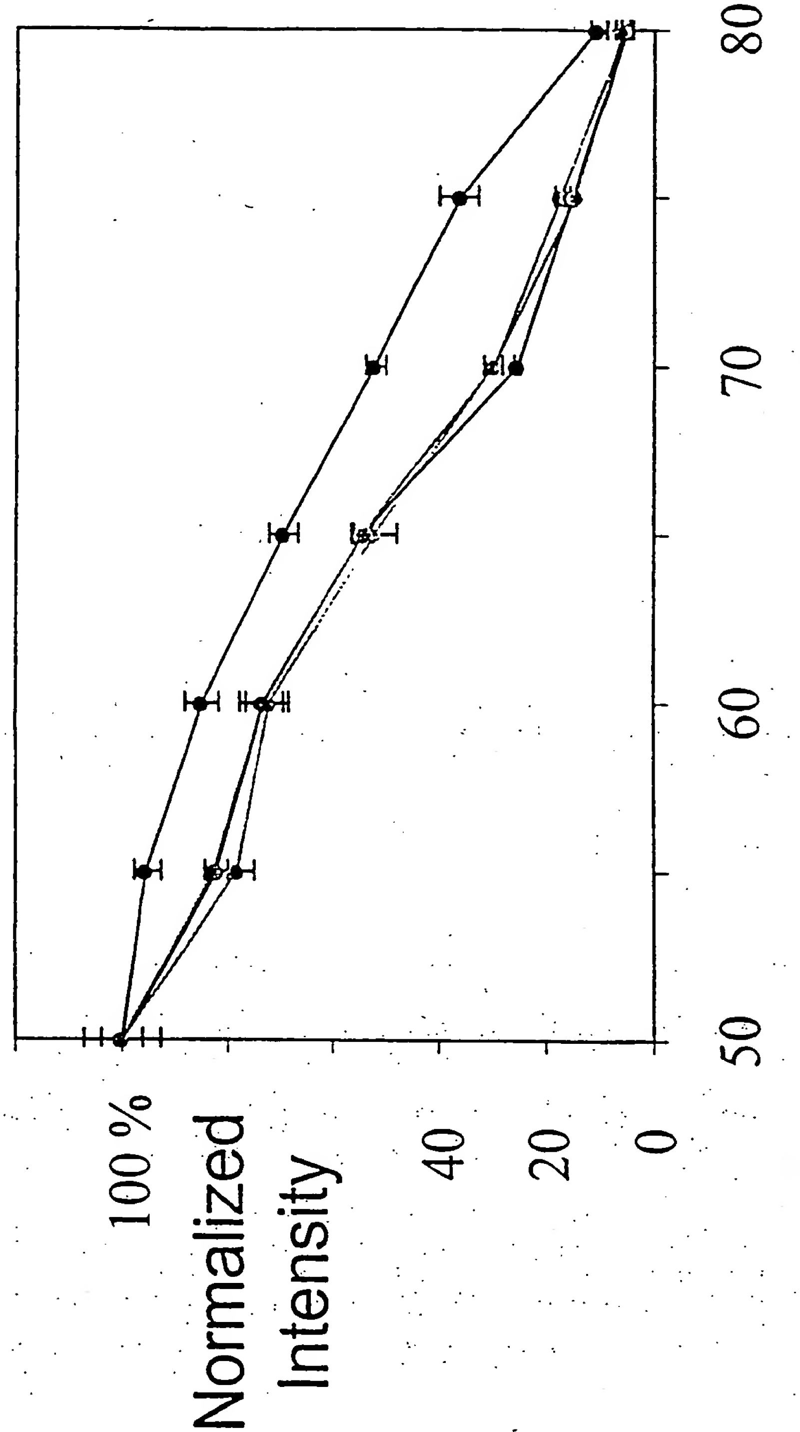

Fig 14

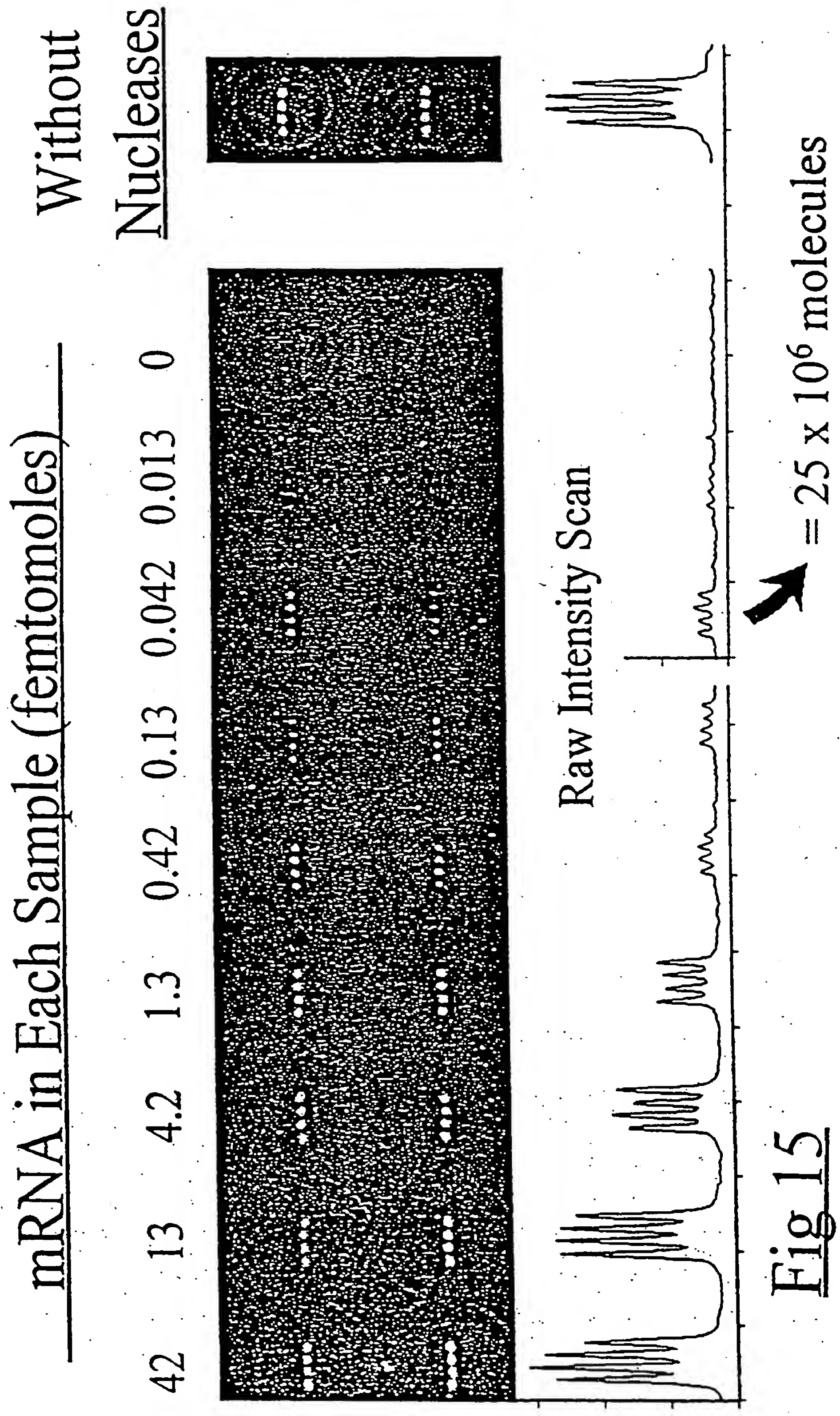
mRNA in Each Sample (femtomoles)
42
13
4.2
1.3
0.42
0.13
0.042
0.013
0
Without
Nucleases
Raw Intensity Scan
= 25 x 10^6 molecules

Fig 15

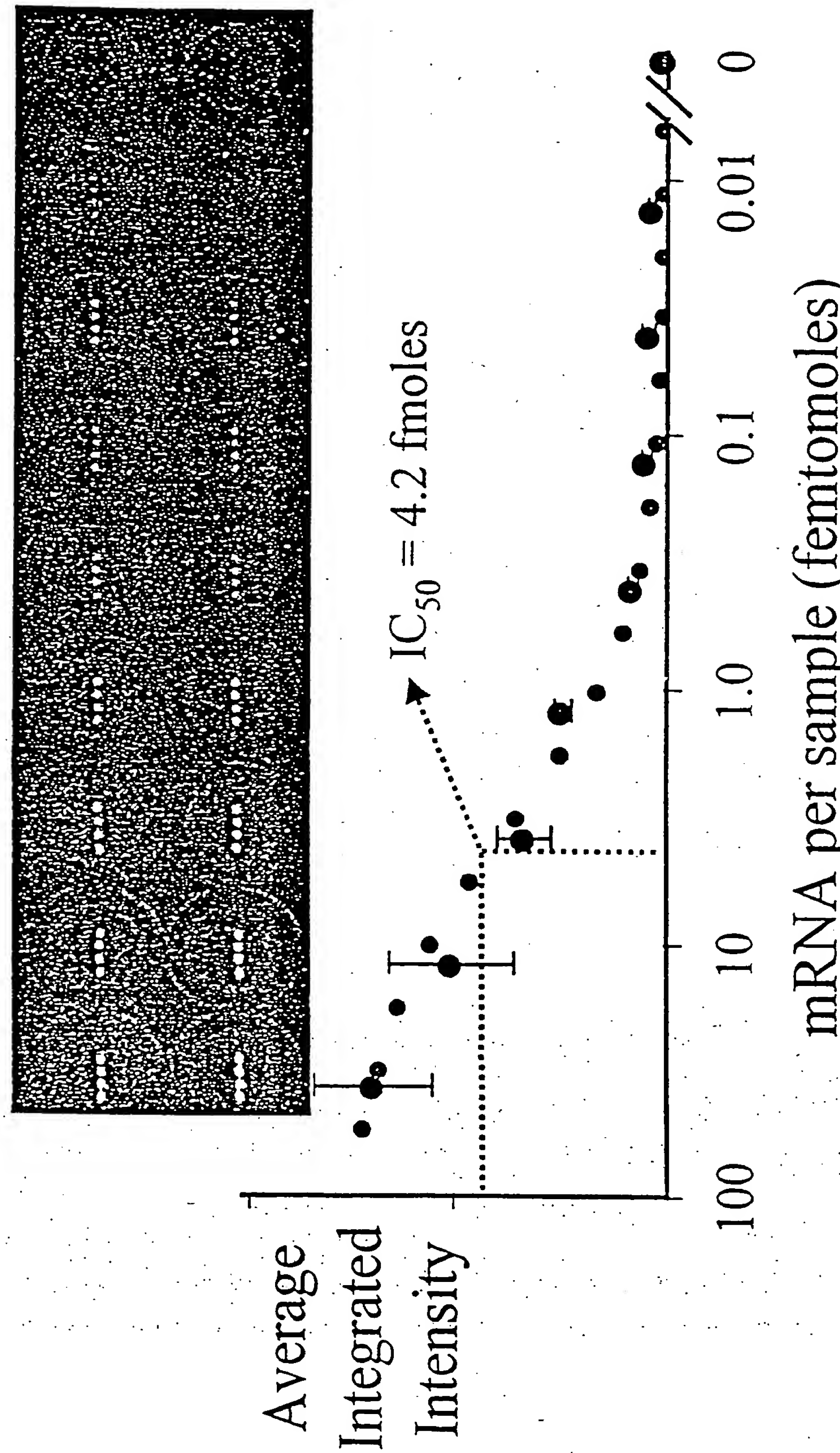
Average Integrated Intensity
IC_{50} = 4.2 fmoles
100
10
1.0
0.1
0.01
0
mRNA per sample (femtomoles)

Fig 16

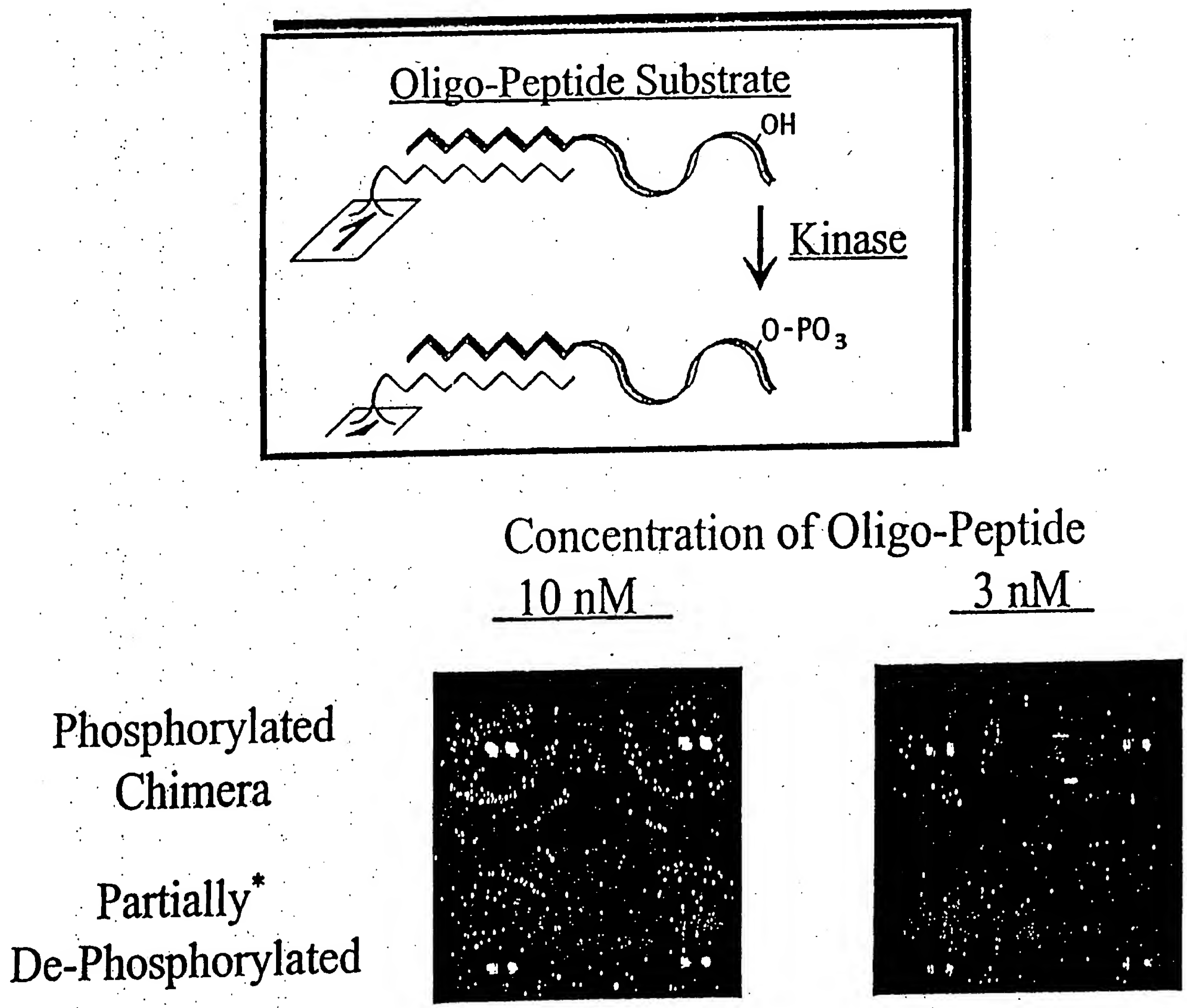
Oligo-Peptide Substrate
OH
Kinase
O-PO3
Concentration of Oligo-Peptide
10 nM
3 nM
Phosphorylated
Chimera
Partially*
De-Phosphorylated

Fig 17

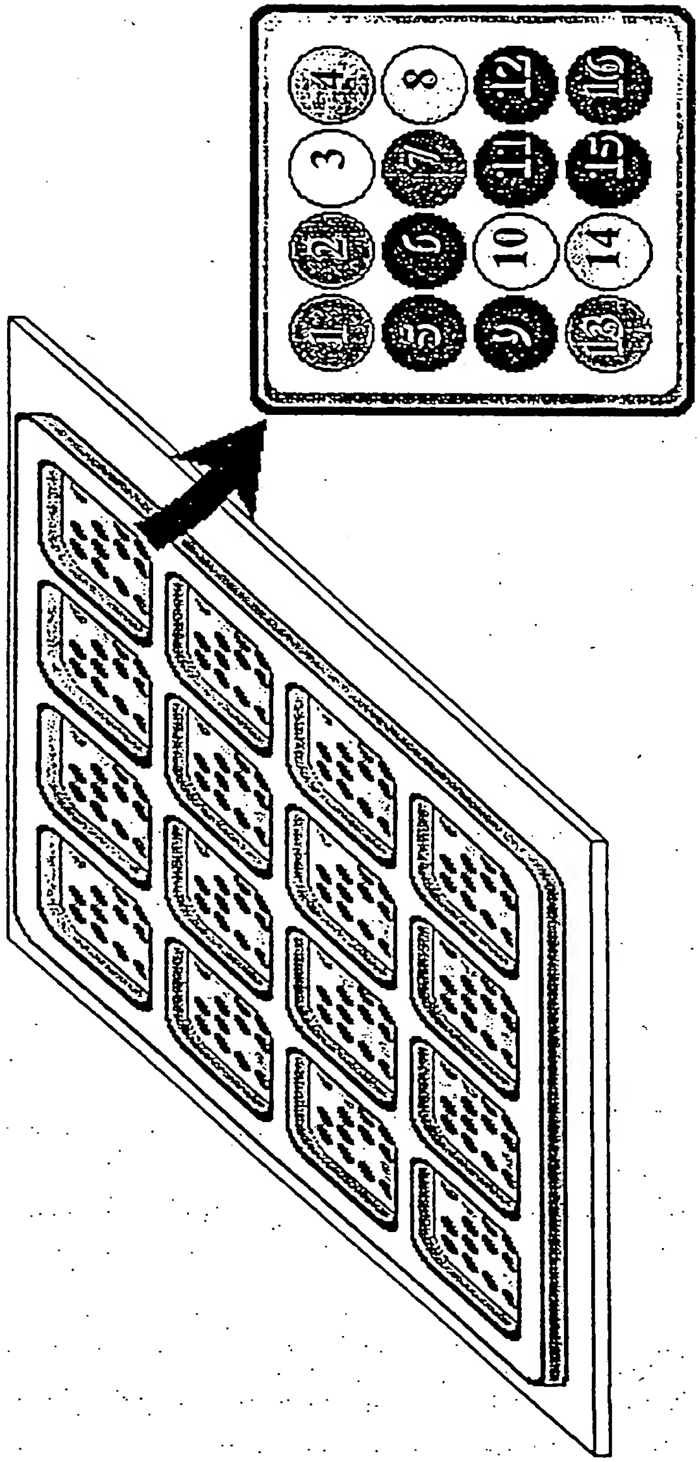

Fig 18

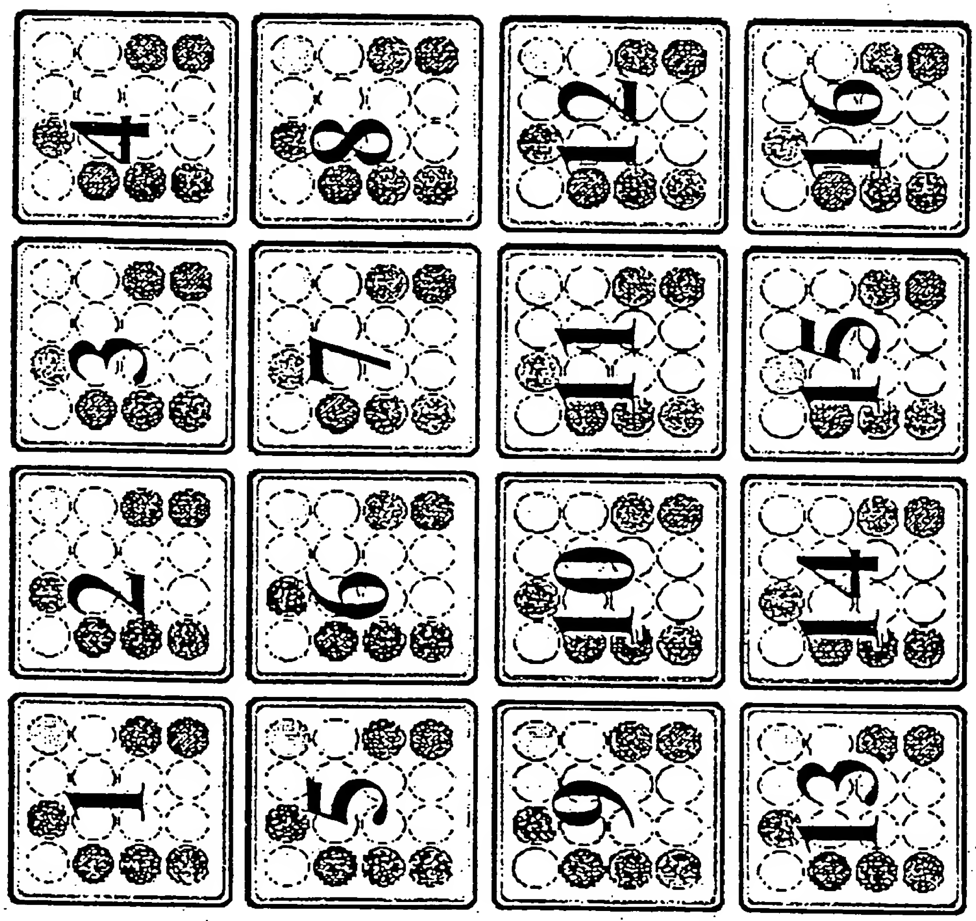

Fig 19

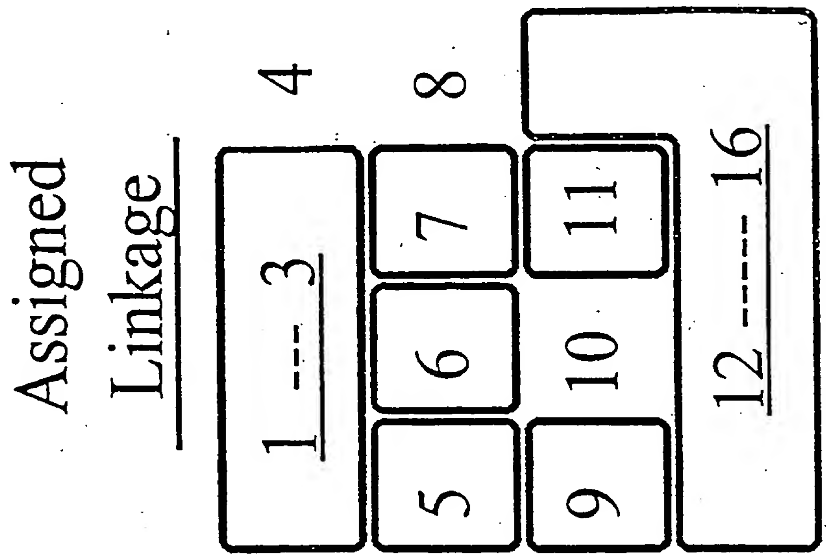

Fig 20b

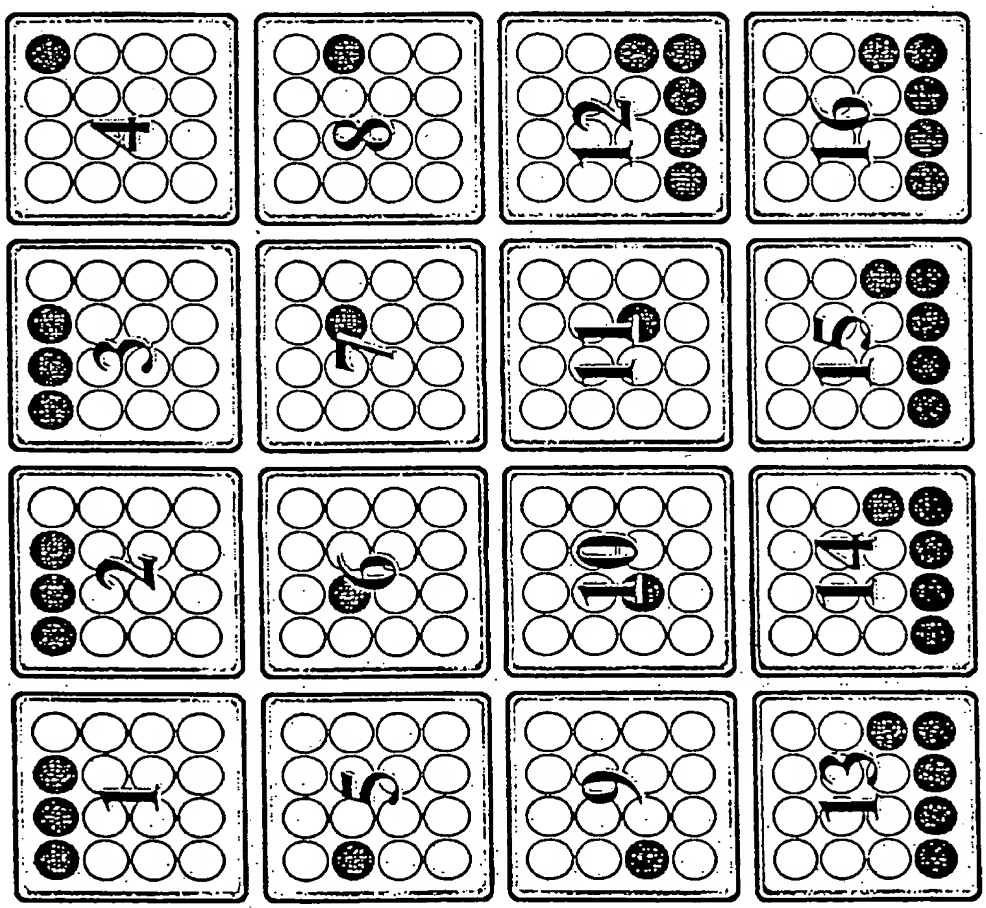

Fig 20a

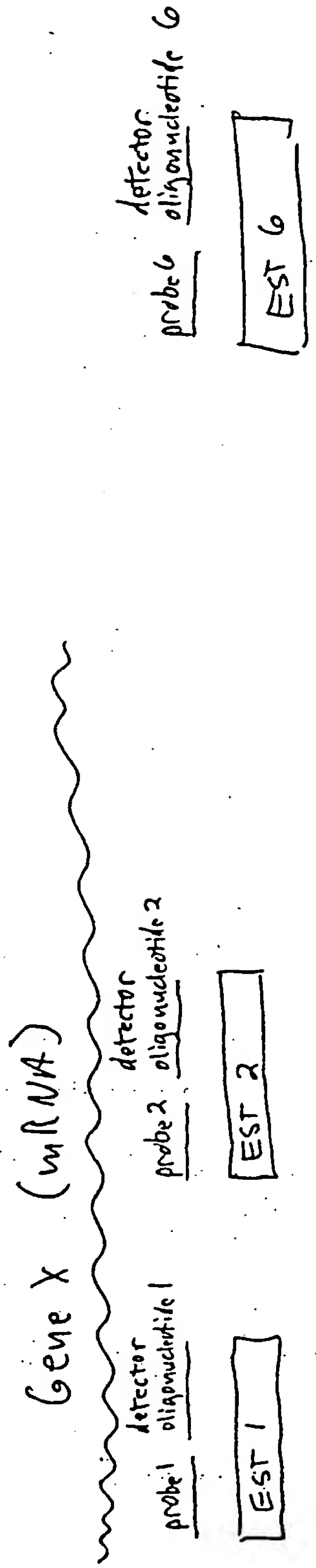
Gene X (mRNA)
probe 1
detector oligonucleotide 1
EST 1
probe 2
detector oligonucleotide 2
EST 2
probe 6
detector oligonucleotide 6
EST 6

Fig 21

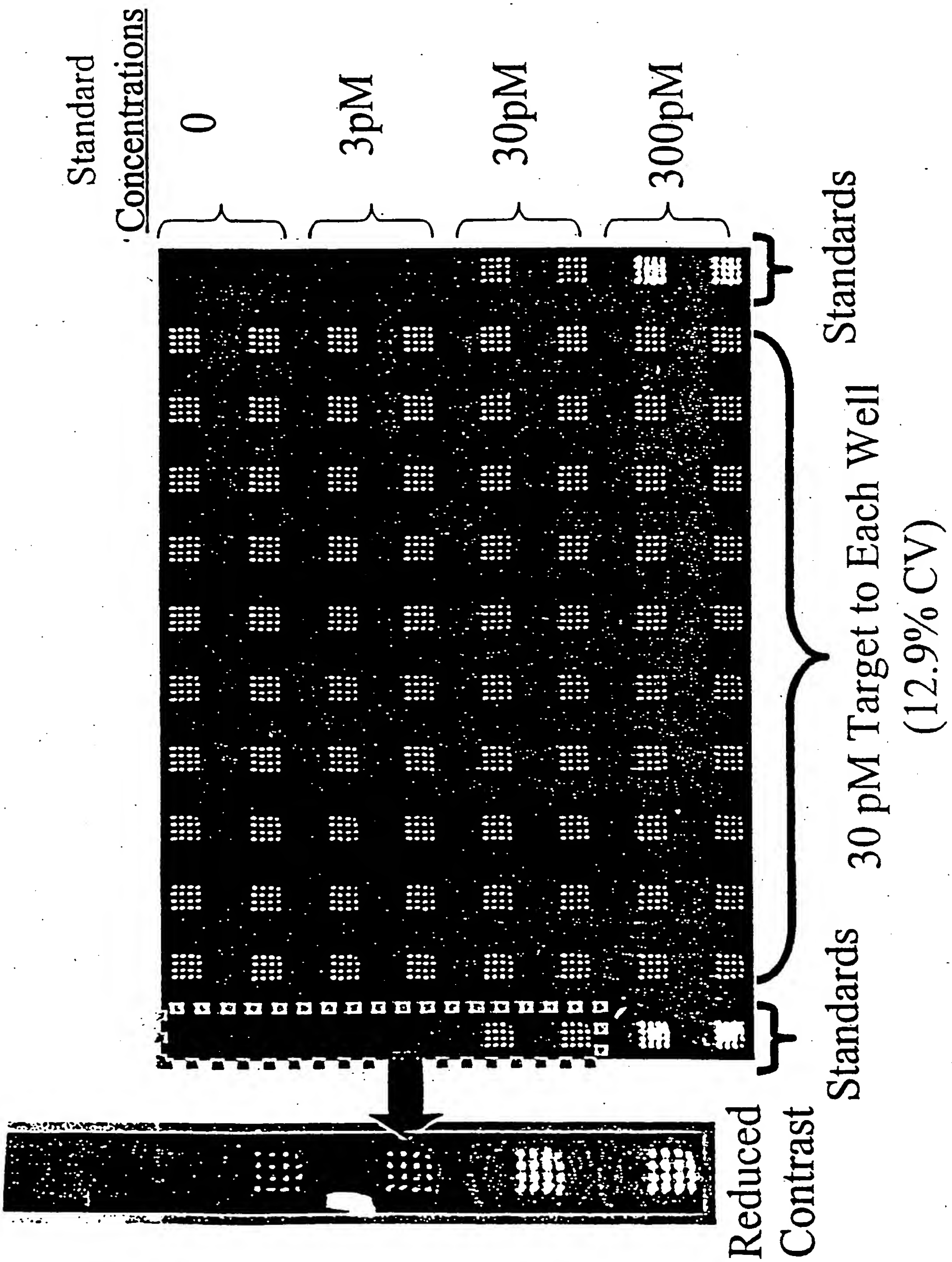
Standard Concentrations
0
3pM
30pM
300pM
Standards
30 pM Target to Each Well
(12.9% CV)
Standards
Reduced Contrast

Fig 22